STUDIES ON

THE ADRENAL CORTEX.

by

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Thesis submitted to the University of Edinburgh for the Degree of Doctor of Medicine.

I N D E X.

PART I. Page

PART I

THE DEVELOPMENT OF AN ASSAY METHOD FOR ACTH BASED ON ITS INHIBITING EFFECT ON THE FORMATION OF GRANULATION TISSUE, AND EXAMINATION OF THE PHYSIOLOGICAL FACTORS AFFECTING THE RESPONSE OF EXPERIMENTAL WOUND HEALING TO ACTH.

INTRODUCTION I

When this work was begun in 1949 there was little relevant literature. ACTH was being assayed generally by one of three methods:-

a). that of Sayers, Sayers & Woodbury (1948) which depends on the depletion of the adrenal ascorbic acid in hypophysectomised rats.

b). the method of Simpson, Evans & Li (1943) which depends on the maintenance of adrenal size in hypophysectomized rats by ACTH.

c). the method of Simpson et al (1943) which depends on the repair of adrenals in hypophysectomised rats by ACTH.

Methods (b) and (c) use much ACTH (an important factor until recently) and all three methods require hypophysectomised rats which is a practical disadvantage in many laboratories. A search for other methods of assay was therefore going on in 1949.

It was noticed independently by Ragan, Grokoest & Boots (1949) and Prunty (1949) that ACTH and cortisone were inhibiting the healing of biopsy wounds in patients. This prompted Ragan, Howes, Plotz, Meyer & Blunt (1949) to study the effect of cortisone on granulation tissue formation in the ears of rabbits. They found that 12.5 mg. of cortisone acetate per day per rabbit for eight to eleven days produced inhibition, but they failed to obtain a good response in rats. It had also been shown by Baker, Ingle, Li & Evans (1948) that the daily injection of 1 - 3 mg. ACTH daily for twenty one

days or 8 mg. daily for ten days interfered with the growth of intact skin; there was thinning of the epidermis, a reduced growth of hair, and the dermal connective tissue became more compact. Baker & Whitaker (1948) also showed that the local application of aqueous adrenal extract to rat skin inhibited the growth of hair and epidermis locally.

It seemed as though the inhibition of healing might be used for the assay of ACTH if smaller laboratory animals, preferably mice, proved suitable. A considerable amount of preliminary work was performed, before a method for consistently obtaining granulation tissue was developed. The assay method has finally used mice, but the technique for obtaining granulation tissue has been found equally useful for studying healing in rats and guinea pigs, and would indeed appear to be applicable to any animal. In the assay a quantal response is measured.

A number of ACTH preparations have been assayed both by this new method and by that of Sayers et al (1948) so that the biological potencies could be compared. It was felt that the inhibition of granulation tissue was possibly more closely related to the effect produced by ACTH and cortisone in rheumatoid arthritis, and therefore might be useful on that account.

In 1949 too, Taubenhaus & Amromin (1949) had begun their studies on the influence of some steroids on granulation tissue. They produced abscesses surrounded by granulation

tissue in rats by injecting turpentine. Testosterone propionate, and oestradiol dipropionate even more, inhibited granulation tissue and led to the production of scanty coarse clumps of collagen. If their animals were pretreated with DOCA for several weeks before the injection of turpentine, granulations formed even more profusely than usual. Following adrenalectomy or castration, the granulation tissue was only slightly inferior in type.

Thus little work had been done on physiological factors concerned in the inhibition of healing by ACTH and cortisone.

In the following pages, experimental work leading to the development of the assay, is described. The potencies of ACTH preparations by this new method and that of Sayers et al (1948) have been compared. An investigation was then undertaken to find out more about the way in which inhibition was produced by ACTH itself. The effects of hypophysectomy, gonadectomy, and adrenalectomy on the response of healing to ACTH were investigated. Since adrenalectomized mice at the beginning of the breeding season, but not later, showed inhibition in response to ACTH, the influence of gonadotrophins was then investigated. This involved experiments on adrenalectomized mice pre-treated with gonadotrophins. It was found that gonads under the influence of gonadotrophins showed a greater response to ACTH. Experiments were then carried out to investigate the effects of some steroids on healing, including the response of intact and gonadectomized mice to cortisone. The healing of baby mice has also been examined. The healing technique has been applied to guinea pigs too, and attempts made to inhibit healing with cortisone.

EXPERIMENTAL I

EXPERIMENTAL

THE HEALING TEST.

ATTEMPTS TO PRODUCE GRANULATION TISSUE.

Using mice of three strains, hamsters and rabbits, various techniques were tried in an attempt to produce granulation tissue consistently and rapidly. These experiments are summarized in the order in which they were performed in the following table;-

Animal used	Technique	Result	Remarks
1. Albino mice; mixed brown mice.	Turpentine was injected beneath the plantar aponeurosis.	After 7 days abscess formation had occurred, but vascular granulation tissue was present in some areas only; no dressing was applied	Since 7 days were required to give abscess formation and as the amount of granulation tissue produced was small and variable, this method was considered unsatisfactory.
2. Albino mice; mixed brown mice.	Two subcutaneous injections of turpentine or 4% formaldehyde were made at the same site on the anterior abdominal wall with a five day interval between injections.	Small amounts of granulation tissue were present after 11 days, but the response to these irritants was irregular; no dressing was applied.	This method was considered unsatisfactory

Animal used	Technique	Result	Remarks
3. Pure black mice.	A piece of skin was removed from the anterior abdominal wall and teak sawdust rubbed into the wound; no dressing.	Vascular granulation tissue began to form in the floor after 3 days. No dressing was applied.	Three days was considered too long a time for a test.
4. Hamsters	0.03 ml. to 0.1 ml. of turpentine was injected intradermally on the anterior abdominal wall.	Abscess formation was rapid, and, sections examined 24, 40 and 48 hours and 6 days after injection showed a consistent production of granulation tissue after 48 hours. No dressing was applied.	This technique was considered possible, but unfortunately the strain of hamster available was very vicious and difficult to handle.
5. Rabbit	Skin was removed from a small area on the outer surface of the ear with sterile precautions, and the area was dressed with sterile vaseline gauze, stitched on to the ear according to the method of Ragan, Howes, Plotz, Meyer & Blunt (1949).	Healthy profuse granulation tissue was formed consistently in $3\frac{1}{2}$ days.	It was concluded that an adaptation of this type of dressing might prove useful in mice.
6. Albino mice.	Skin was removed from the anterior abdominal wall with sterile precautions and the wound was covered with sterile vaseline gauze with or without turpentine impregnation. The dressing was held in place with cotton wool and adhesive tape	After 24 hours, granulation tissue was present in some animals, the response being more valuable when turpentine was present. Wounds contaminated with urine or faeces showed no healing response whatsoever. Some of the dressings came off.	Vaseline gauze was a suitable dressing, but the cotton wool and tape tended to come off and allowed the wounds to become contaminated.

Animal used	Technique	Result	Remarks
7. Albino mice.	The technique finally devised is described below.	Sections made at intervals up to 48 hours after wounding showed that granulation tissue was forming after 9 hours and was consistently present in a satisfactory amount after 28 hours.	In this technique the important points are:- 1) A consistent technique. 2) Asepsis 3) Suitable dressing 4) Interval of 28 hours.

Technique finally adopted.

Male albino mice, bred in our own colony, weighing 12 - 18 g. were maintained on a diet of Thomson cubes and water. Operation was performed under light ether anaesthesia and an aseptic technique was adopted as far as possible. Instruments were sterilised by boiling. Fur was removed from the anterior abdominal wall with scissors, and a piece of skin about 2 to 3 mm. in diameter in the mid-line of the anterior part of the ventral surface of the abdomen was lifted with fine forceps and clipped off with sharp scissors. The wound was covered with three thicknesses of sterile vaseline gauze, over which was placed the cap of a bottle about 15 mm. in diameter. The dressing was held in place with adhesive tape and the cap prevented any pressure on the wound and any contamination with urine and faeces. Care and attention to detail are essential, if consistent controls are to be obtained. Twenty

eight and a half hours later the mouse was killed and the dressing was carefully removed. Some practice was required before the dressings were satisfactory. One inch wide adhesive tape was torn to give strips approx. $\frac{1}{3}$ inch wide, as these strips showed more elasticity than narrow tapes. Tape was manufactured by Dalzo or Johnsons was more adhesive than other brands. The tape must be applied firmly but not so tightly that it impedes respiration, or the blood supply to the wounded area.

Appearance of healing tissues.

Two to three minutes after removal of the dressing, the ulcers were examined about two feet beneath and one foot to the side of an electric light. The appearance of a healing ulcer is shown in figs. 1 and 2 . It showed marked hyperaemia, sloping margins, a rough thickened floor and the original vessels in the floor were obscured by new tissue.

Histological examination of most ulcers was carried out. At first, difficulty was experienced in obtaining satisfactory sections; if the skin was merely immersed in formalin it curled up, and if pinned out on cork in formalin the granulation tissue was torn; It was found that satisfactory sections were obtained if the ulcer and a large piece of the surrounding skin was removed and placed flat on a thick piece of blotting paper. After one minute the skin and paper were immersed in formalin. The paper was removed twenty four hours later, and

Figure 1. Left, control healing ulcer; right inhibited ulcer.

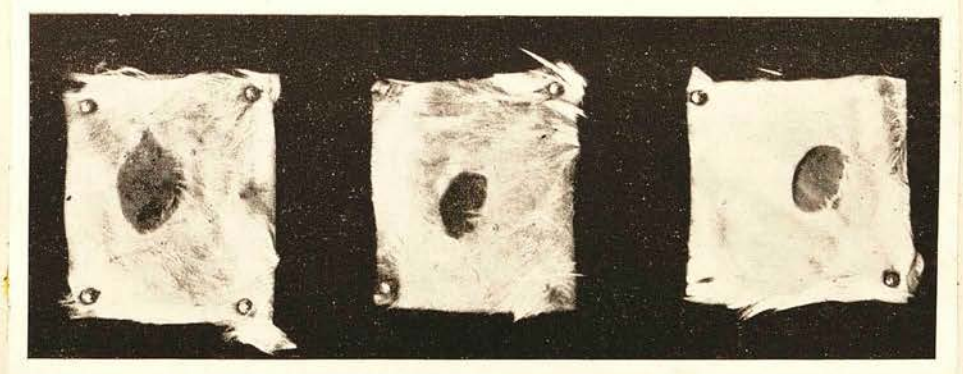


Figure 2. Left, control healing ulcer; centre partly inhibited ulcer; right completely inhibited ulcer.

Figure 1. Left, control healing ulcer; right inhibited ulcer.

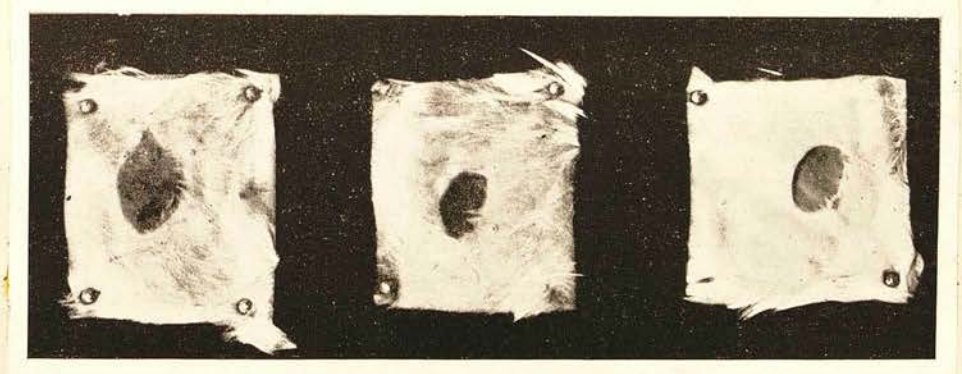


Figure 2. Left, control healing ulcer; centre partly inhibited ulcer; right completely inhibited ulcer.

the skin was trimmed. Since the ulcers were to be sectioned in the transverse diameter, a dot on the skin was made with Indian ink on either side of it, so that the sections required were easily seen when the wax block was being cut with a microtome. (see fig. 3.).

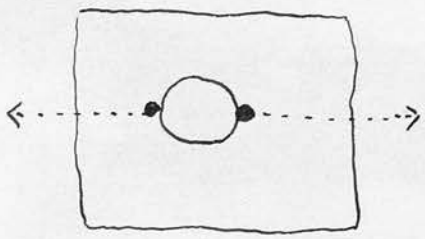


Fig. 3.

Sections were stained with haematoxylin and eosin. The histological appearances are shown in figs. 4,5 and 6 .

It is found that the thick floor of the control ulcer (fig. 5.) consists of granulation tissue with marked polymorph infiltration, proliferating fibroblasts and numerous capillaries.

Effect of ACTH

In an attempt to use as little ACTH as possible, owing to extreme shortage of material when this work was begun, a multiple injection technique was adopted. By a fortunate chance the first schedule dosage tried was successful, and it has been found impossible to improve on it.

The ACTH was given in normal saline as twenty five subcutaneous injections, each of 0.1 ml. Injections were given one hour and a half an hour before the ulcer was made in the

Figure 4.

Section of ulcer immediately it was made. x 42

Figure 5.

Section of normally healing ulcer x 42

Figure 6.

Section of completely inhibited ulcer. x 42.

morning, immediately after making it and then hourly except between 11.30 p.m. and 7.30 a.m., when only two injections were given.

In preliminary experiments, 1.8 mg. of ACTH (Armour H.7911) were given and complete inhibition of healing was obtained. The inhibited ulcer showed no hyperaemia, had sharp clear-cut margins and a smooth thin floor in which the original vessels were still visible, (fig. 1.). Microscopically the floor of such an ulcer was very thin, and contained only a few polymorphs, some flattened fat cells and no capillaries (fig. 6).

The macroscopic appearance of a healing ulcer and an inhibited ulcer are summarised as follows:-

Control healing ulcer	Inhibited ulcer
1. Marked hyperaemia.	1. No hyperaemia
2. Sloping margins.	2. Sharp, clear-cut margins; this appearance is emphasised by the shadow in the third lesion (fig 2).
3. Rough, thick floor	3. Smooth, thin floor.
4. The original vessels in the floor are obscured by new tissue.	4. The original vessels in the floor are still visible.

Attempts to simplify dosage.

Attempts were made to reduce the number of injections, and particularly to exclude the two doses given during the night. 45 µg. of H.7911 which by the twenty five injection technique consistently produced inhibition in half the mice, failed to cause complete inhibition in any if the total number of injections was cut by a third or a half, even though the total amount given at 9.30 and 10.30 p.m. before the gap during the night was increased by as much as four times. Also, increasing the total dosage of H.7911 to 1.0 mg. per mouse failed to give complete inhibition when the night doses were excluded. It therefore became apparent that regular injections throughout the twenty eight and a half hours were essential.

It was also found that the ulcers must be cut before mid-day so that a sufficient length of time elapses between cutting and the more widely-spaced doses of the night.

In a further attempt to reduce the number of injections, 65 µg. H.7911 was placed in 0.2 cc. "Subtosan". 0.1 cc. was given one hour before and again six hours after cutting. In a group of four mice healing was impaired in one only. Subtosan alone did not impair healing. It is possible that further work might produce a result, but it seems unlikely, as the action of ACTH in patients was not very prolonged by using Subtosan as a vehicle.

An attempt was also made to prolong the action of ACTH by administering it in the form of an implant having a lipoid base. The implants were made by Professor J.M. Robson. They were placed in the loose subcutaneous tissues at the back of the neck through an incision made under light ether anaesthesia, one and a half days before the ulcers were made. Anaesthesia and implantation impair healing and so one and a half days were allowed to elapse before cutting. The results were as follows:-

	Number of mice.	Dose in µg. La - 1 - A.	Response		
			Inhibited	Impaired	Healing
a)	6	312.5 (1 implant weighing 1 mg.)	0	3	3
b)	4	625 (2 implants)	0	3	1

In group (b) the implants were recovered at the end of the experiment, dried and weighed. It was calculated that the absorption per mouse was equal to 275 µg. La-1-A. This was a large dose, and complete inhibition was not produced in any animal. It was concluded that the method was not sufficiently satisfactory to justify the use routinely of such a large amount of ACTH, and the method was abandoned.

In addition, healing was studied eight hours after wounding with a range of mice varying in weight, from 12 - 30 g using appropriately higher doses of ACTH. Though granulation tissue was frequently present in large amounts after eight hours in mice weighing 25 - 30 g., its formation was not

consistent enough to be made the basis of a test which could be completed during one normal working day.

In an attempt to increase the number of mice available for tests, male albino mice bred in colonies at Boots and Glaxo were studied. Though healing was apparent in twenty eight hours it was not sufficiently advanced to be made the basis of a macroscopic test. Since twenty eight hours was already considered sufficiently long for the test, these two strains were not used again.

An attempt was made to increase the number of mice by using females as well as males. Unfortunately the females healed more slowly, and granulation tissue was not consistently present at twenty eight and a half hours. At least thirty six hours were needed and this was considered too long for a test.

ASSAY METHOD BASED ON WOUND HEALING.

After much consideration, it was decided that the measurement of a quantal response was most practical. The size of the wound, the thickness of the granulation tissue in the floor and the slope of the margins of the wound all showed too much variation even in control animals to be made the basis of an assay depending on a graded response to a given series of doses. Even if the variations had not been so large, the labour necessary to section every wound in an assay would have made the method impracticable.

In the assays, a preparation of ACTH, Armour H.7911, was used as a laboratory standard. Doses of this, ranging between 33 and 62 µg. were given to groups of ten mice. The percentage healed in a group was plotted against the logarithm of the dose and gave a sigmoid curve as shown in fig. 7 . A probit line was fitted and it was calculated that the approximate 95 per cent limits of error would be 78 to 128 for ten animals in each of two preparations, 84 to 119 for twenty animals and 90 to 112 for fifty animals. The actual results on which fig. 7. is based are as follows:-

Dose in µg.	Number of animals	Number of animals healing.
33.7	9	8
37.7	9	7
41.2	9	7
45.5	10	5
50.2	7	2
55.5	9	1
61.3	8	0

The plan of an assay was as follows:- Groups of four animals were placed on four doses of standard and four of unknown, giving a total of 32 animals, per assay; it was considered that this would be a compromise between reasonable limits of error and the number of animals required for an assay.

Assays performed by this method have been compared with those obtained by the method of Sayers, Sayers & Woodbury (1948), which depends on the depletion of ascorbic acid in in the adrenals of hypophysectomised rats. Their technique has been slightly modified and is described later.

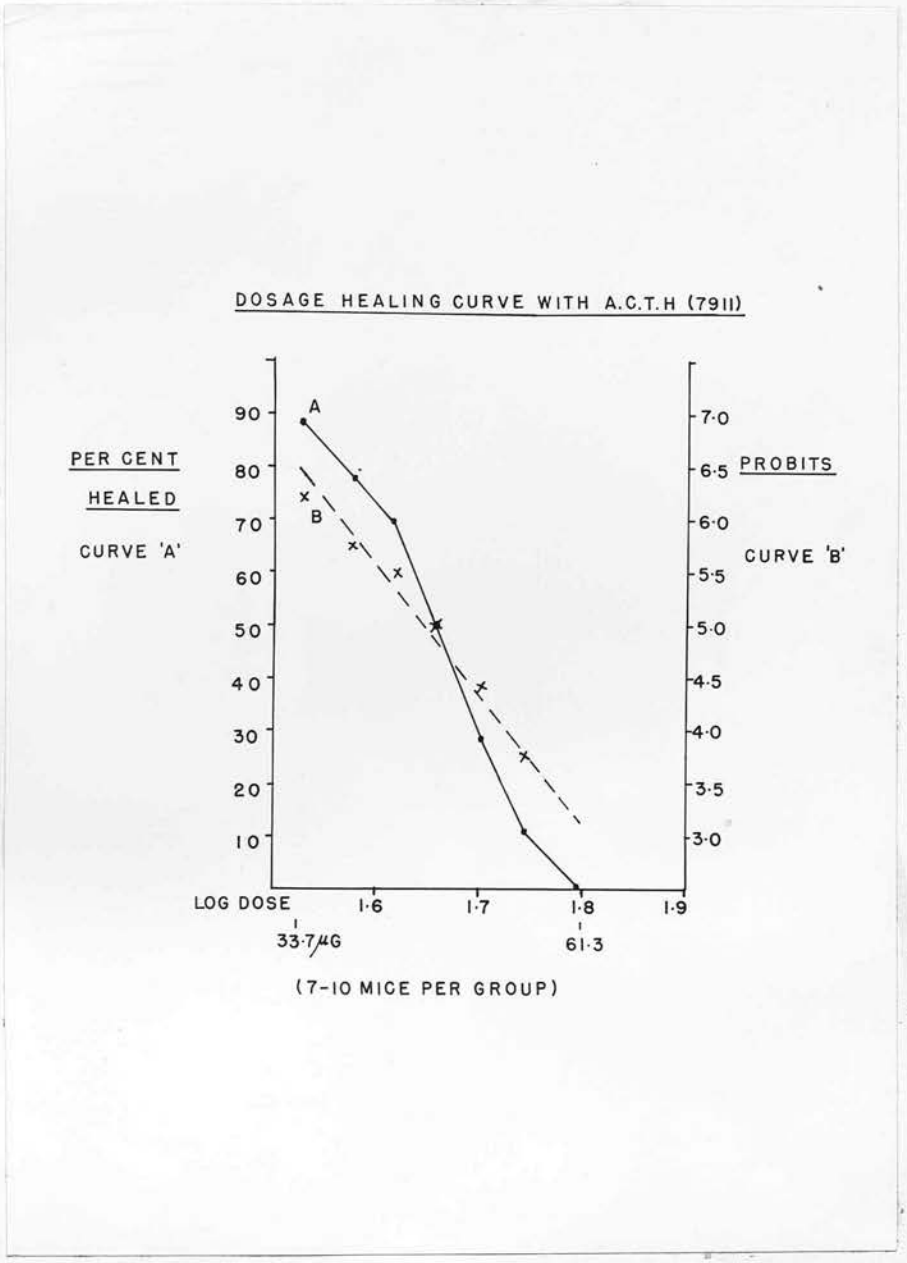


Figure 7. Dosage healing curve with standard ACTH (H.7911)

The results of assays on two preparations of ACTH in 1951, one from Organon and one made by Dr. Reiss are shown in Table 1 , and comparative figures with the Sayers' test are given.

Table 1.

ORGANON			REISS		
Dose in µg.	Number of animals	% healed	Dose in µg.	Number of animals	% healed
418.0	4	75	135.0	4	100
469.0	4	75	151.5	4	50
526.0	4	50	169.9	4	50
590.5	4	50	190.6	4	25.

Standard: H.7911/Organon = 0.083 (Sayers' method 0.070; Limits 75% to 132%) Limits (p = 0.95) = 0.071 to 0.097.

Standard: H.7911/Reiss = 0.265 (Sayers' method 0.234; Limits 46% to 212%) Limits (p = 0.95) = 0.228 to 0.309.

The healing assay has been carried out on eight ACTH preparations (Armour)during the past year (1952). In every instance a standard has been run at the same time.

The results were as follows:-

H.7911		Unknown	
Dose in µg.	Number of mice inhibited in each group	Dose in µg.	Number of mice inhibited in each group
41.2	1 out of 4	123.5	1 out of 4
50.2	2 " " 4	150.8	2 " " 4
60.3	3 " " 4	183.9	2 " " 3
74.8	1 " " 3	224.5	3 " " 4
		J.28112	
41.2	0 " " 3	247.0	1 " " 2
50.2	0 " " 4	301.5	1 " " 4
60.3	1 " " 2	361.8	0 " " 3
74.8	2 " " 3	448.8	2 " " 4

H.7911		Unknown	
Dose in µg.	Number of mice inhibited in each group	Dose in µg.	Number of mice inhibited in each group
		J.20507	
41.2	1 out of 4	41.2	2 out of 4
50.2	1 " " 4	50.2	2 " " 4
60.3	2 " " 4	60.3	1 " " 3
74.8	1 " " 3	74.8	3 " " 4
		J.25711	
50.2	2 " " 4	245.2	1 " " 4
60.3	3 " " 4	299.2	2 " " 3
74.8	2 " " 3	365.0	3 " " 4
91.3	1 " " 2	445.6	2 " " 3
		K.54006	
33.7	1 " " 4	246.0	1 " " 4
41.2	1 " " 4	301.5	2 " " 4
50.2	1 " " 4	367.8	2 " " 4
60.3	2 " " 4	448.8	2 " " 4
		J.10602	
50.2	2 " " 4	41.2	0 " " 4
74.8	2 " " 4	50.2	2 " " 4
		60.3	3 " " 4
		74.8	4 " " 4
		K.50802	
33.8	1 " " 4	123.5	0 " " 4
41.2	1 " " 4	150.7	0 " " 4
50.2	1 " " 4	183.9	2 " " 2
60.3	2 " " 3	224.5	1 " " 4
		224.5	1 " " 4
		224.5	2 " " 4
		273.0	2 " " 2
		H.7010	
		1010.0	1 " " 2
		1230.0	3 " " 4
		1500.0	3 " " 3
		1800.0	3 " " 3

These results have been submitted to Mr. P. Armitage for analysis. He has reported that "on any particular day the slope would not be significant, and the only reasonable thing to do is to use a pooled slope from the whole data (i.e. a sort of average of the separate slopes), verifying that there is no evidence of heterogeneity between the separate slopes.

This works out satisfactorily. The pooled slope is 4.23 with a standard error of 0.95, and, although significant, is much smaller than the slope of about 12 which you got before. The potencies of the unknowns in terms of H.7911 are as follows:-

Unknown	Estimated potency	95% confidence limits
K.29901	0.36	0.22 - 0.61
J.28112	0.16	0.09 - 0.29
J.20507	1.33	0.80 - 2.25
J.25711	0.18	0.11 - 0.31
K.50802	0.24	0.15 - 0.39
H.7010	0.072	0.041 - 0.135
K.54006	0.16	0.10 - 0.27
J.10602	1.10	0.61 - 2.01

Apart from the use of a pooled slope, each day has been regarded as providing a self-contained assay. The tests for (a) differences between slopes, and (b) departures from linearity of the individual slopes, give

(a) χ^2 = 13.2 on 14 d.f. $(0.5 < P < 0.7)$

(b) χ^2 = 17.7 on 29 d.f. $(0.90 < P < 0.95)$

so our assumptions are at least not contradicted.

Since the previous slope was nearly three times the present one, almost $3^2 = 9$ times as many animals will now be required to obtain the same degree of accuracy as before."

I think the change of slope may be due to beginning the assays later in the morning, so that the night injection period occurs earlier during the healing period.

These prepararations have also been assayed by the Sayers' test, and in this H.7911 has 6.13 times the potency of La-1-A. The potency of these preparations against H.7911 is as follows:-

K.29901	unsatisfactory assay but less than 1 x La-1-A.
J.28112	0.16
J.20507	1.39
J.25711	0.33
K.50802	0.33
H.7010	0.035
K.54006	0.16
J.10602	1.06

The limits of error for the Sayers' test are 0.50 to 2.00 in our hands.

The potencies as determined by the two methods do run parallel to each other. Unfortunately there has not been enough La-1-A available to assay it against H.7911 in the healing test.

ADRENAL ASCORBIC ACID DEPLETION METHOD.

Technique.

Wistar male rats weighing 75 to 130 g. were brought into the laboratory at least three days before use. They were fed ad lib. on Rowett diet 46 (Obtained from Haygate & Sons) before operation and afterwards on bread and milk. Attempts to maintain their environment at 70° to 75°c. were not always successful, and when sudden marked fluctuations occurred the techniques of hypophysectomy and adrenalectomy were made more difficult as blood clotting was sometimes impaired. Hypophysectomy was performed under ether anaesthesia. The freshness of the ether was found to be most important if respiratory difficulties were to be avoided. The parapharyngeal approach was

used and largely by feel, the initial hole in the skull was made with a fine pair of forceps and enlarged with a blunt probe, the gland then being removed by suction. A tracheotomy tube was not necessary after some experience. Any mild bleeding was stopped by applying industrial spirit. At autopsy the sella was examined for completeness of hypophysectomy.

The left adrenal gland was removed under ether anaesthesia 18 to 21 hours later. The solution being assayed was injected into the exposed left-external iliac vein and one hour later the right adrenal was removed. Excised adrenals were cleaned, weighed to the nearest 0.1 mg. on an analytical balance and transferred to 6% trichloracetic acid. Ascorbic acid was determined by the method of Roe & Kuethner (1943). New bottles of trichloracetic acid should be tested with pure ascorbic acid before use as some samples give a precipitate during the final colour reaction.

Soluble preparations of ACTH were injected in normal saline, less soluble ones were taken up in glacial acetic acid and diluted to 0.02 to 0.01 N as suggested by Morris (1950). The solutions were allowed to stand during the tests in a beaker of cold water standing on a tray of ice. Doses were spaced logarithmically and the doses given to particular rats were randomized by picking the doses out of a box.

Results.

The strain of rat used for assay appeared to be most important. The Sprague - Dawley rats in our colony showed great variation in the sizes of adrenal glands, the difference in an individual rat occasionally amounting to as much as 20mg.

Examples of Adrenal weights in mg. in Sprague-Dawley rats.

Right	Left
6.5	18.5
19	19.5
18	26.5
15.7	17
14	13.5

In our Wistar rats the adrenal glands usually weighed 7 - 12 mg. In a series of 150 wistars, the difference was less than 2 mg. in 90% and the right gland was larger than the left in 23%. The ascorbic acid concentrations in the adrenals of hypophysectomized but otherwise untreated rats are given in Table

Some of the percentage differences are much larger than those observed by Sayers et al (1948). Where this occurs it is due to the considerably higher concentration of ascorbic acid in the second gland (right).

TABLE 2.

A random selection of the ascorbic acid concentration of the adrenals of untreated hypophysectomized wistar rats.

Adrenal ascorbic acid mg. per 100 g.		Difference in mg. (left minus right.	Difference per cent of left adrenal.
Left	Right		
438.2	420.4	+17.8	4.0
404.7	402.9	+1.8	0.4
365.8	426.6	-60.8	16.7
494.8	500.0	-5.2	1.0
367.0	346.0	+21.0	5.7
440.0	454.6	-14.6	3.3
565.0	568.4	-3.4	0.6
600.0	583.0	+17.0	2.8
680.8	642.6	+38.2	5.6
644.2	615.2	+29.0	4.5
715.0	721.8	-6.8	0.9
650.0	623.0	+27.0	4.1

Average: 4.1

Standard error: ± 1.62

Owing to the small size of the colony, body weights of the rats were not as uniform as we wished. There were also some severe fluctuations in the temperature of the laboratory on occasion. Three sets of figures were submitted to Mr. Armitage of the M.R.C. Statistics unit; he carried out an analysis of variance and showed that the body weight and initial concentration of ascorbic acid (which probably bears some relation to environmental temperature) had no significant effect on the potency of ACTH determined. The figures submitted to him were as follows:-

Table 3.

a) Preparation of ACTH - Organon 200 : 365

Temperature of room during previous 48 hrs. 66 - 86°F.

Rat No.	Dose in µg.	Body weight in g.	Left adrenal in mg.	Right adrenal in mg.	Concentration of adrenal ascorbic acid (mg. per 100g. tissue). Left	Right	Difference
A	0.6	133	9.3	8.4	464.4	435.6	-28.8
B	2.4	128	10.4	10.3	505.6	316.4	-189.2
C	0.6	115	10.5	7.7	500.8	475.2	-25.6
D	0.6	81	7.2	5.2	572.2	500.0	-72.2
E	1.2	94	7.8	6.7	538.4	456.6	-81.8
F	2.4	115	9.2	6.9	469.4	365.2	-104.2
G	2.4	142	8.4	7.1	523.8	366.0	-157.8
H	0.6	113	9.8	8.8	489.6	445.4	-44.2
J	2.4	122	9.0	6.3	568.8	485.6	-83.2
K	1.2	121	9.1	9.0	643.8	480.0	-163.8
L	2.4	118	8.9	8.5	591.0	428.4	-162.6
M	1.2	117	9.4	8.8	531.8	468.0	-63.8
O	1.2	88	6.7	6.8.	486.4	441.0	-45.4
			Dose:	0.6 µg.	1.2 µg.	2.4 µg.	
			Mean difference:	-42.7	-88.7	-139.4	

Table 4.

b) Preparation of ACTH - Z_3 (Prepared by Dr. Reiss).

Temperature of room during previous 48 hours - constant at 75° F.

Rat No.	Dose in µg.	Body weight in g.	Left adrenal in mg.	Right adrenal in mg.	Concentration of adrenal ascorbic acid (mg. per 100 g. tissue).		Difference
					Left	Right	
A	1.2	134	8.0	6.2	540.0	309.6	-230.4
C	0.6	123	9.1	7.9	650.4	488.6	-161.8
D	2.4	111	8.4	7.5	657.8	496.0	-161.8
F	1.2	110	6.8	6.8	694.0	429.4	-264.6
G	0.6	114	10.4	9.8	626.8	531.6	-95.2
H	2.4	124	8.7	7.7	643.6	423.2	-220.4
I	0.6	77	7.2	5.4	388.8	281.4	-107.4
K	2.4	100	7.7	7.2	605.0	286.0	-319.0
L	1.2	146	10.2	8.4	554.8	380.8	-174.0
M	1.2	125	8.3	7.3	626.4	547.8	-78.6
N	0.6	113	7.4	6.4	567.4	468.6	-98.8

Dose:	0.6 µg.	1.2 µg.	2.4 µg.
Mean difference:	-115.8	-186.9	-233.7

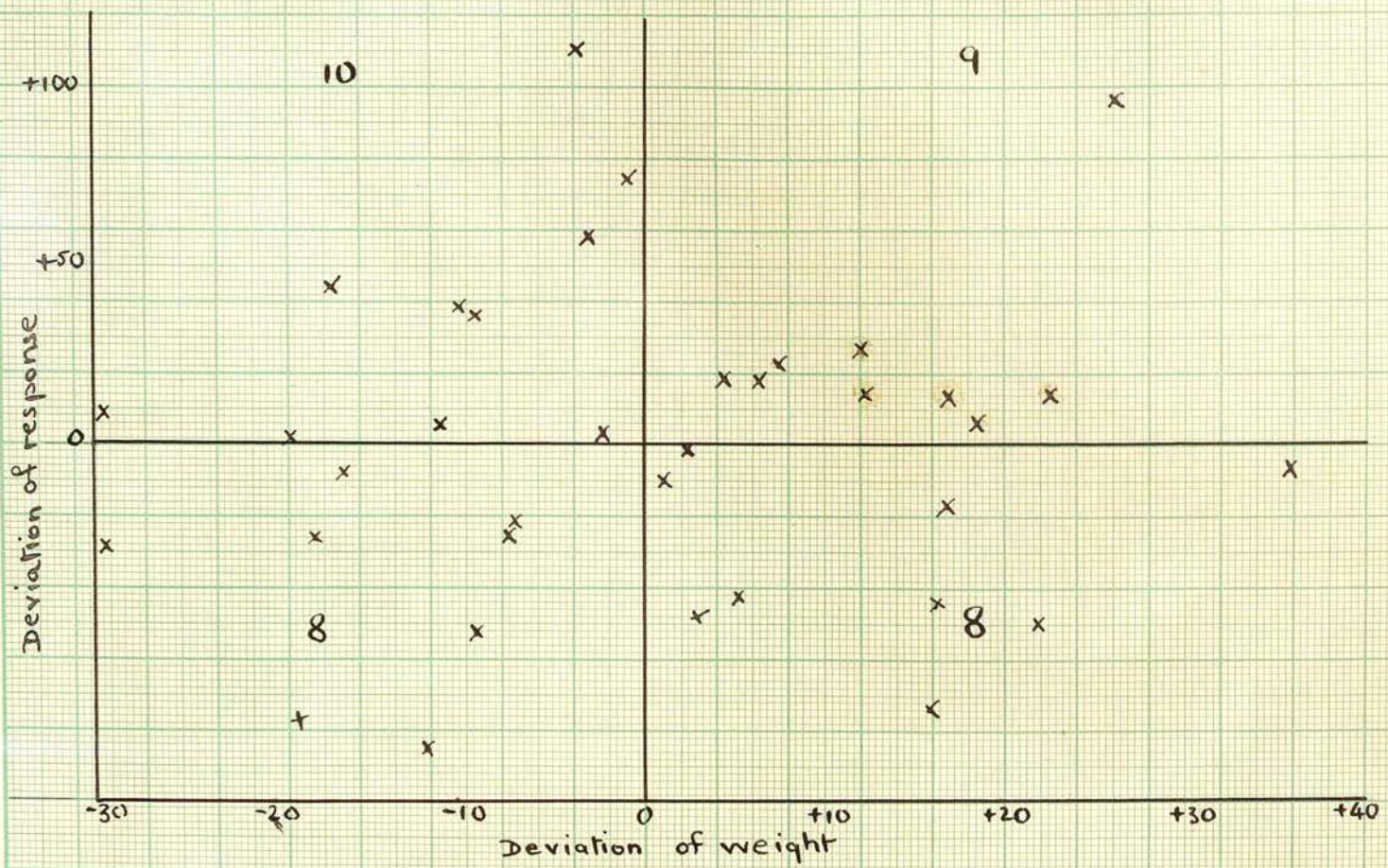
Table 5.

c) Preparation of ACTH - Organon 203 : 935

Temperature of room during previous 48 hrs. 67-78° F.

Rat No.	Dose in µg.	Body weight in g.	Left adrenal in mg.	Right adrenal in mg.	Concentration of adrenal ascorbic acid (mg. per 100 g. tissue).		Difference
					Left	Right	
A	2.4	92	6.0	6.0	500.0	320.0	-180.0
C	1.2	77	6.9	4.6	423.0	373.8	-49.2
D	2.4	83	6.0	5.2	466.6	315.2	151.4
G	0.6	95	6.8	5.4	420.4	355.4	-75.0
H	0.6	93	5.9	4.9	528.8	514.2	-14.6
I	0.6	138	8.6	7.8	618.6	564.0	-54.6
J	1.2	63	5.8	4.2	631.0	571.4	-59.6
K	1.2	98	6.9	6.1	405.8	360.6	-45.2
M	2.4	127	7.4	5.3	494.4	464.0	-30.4
N_1	2.4	102	6.2	5.5	472.2	336.2	-136.0
D_1	0.6	83	7.2	6.4	555.4	509.2	-46.2

Dose:	0.6 µg	1.2 µg.	2.4 µg.
Mean difference:	-42.7	-51.3	-124.4



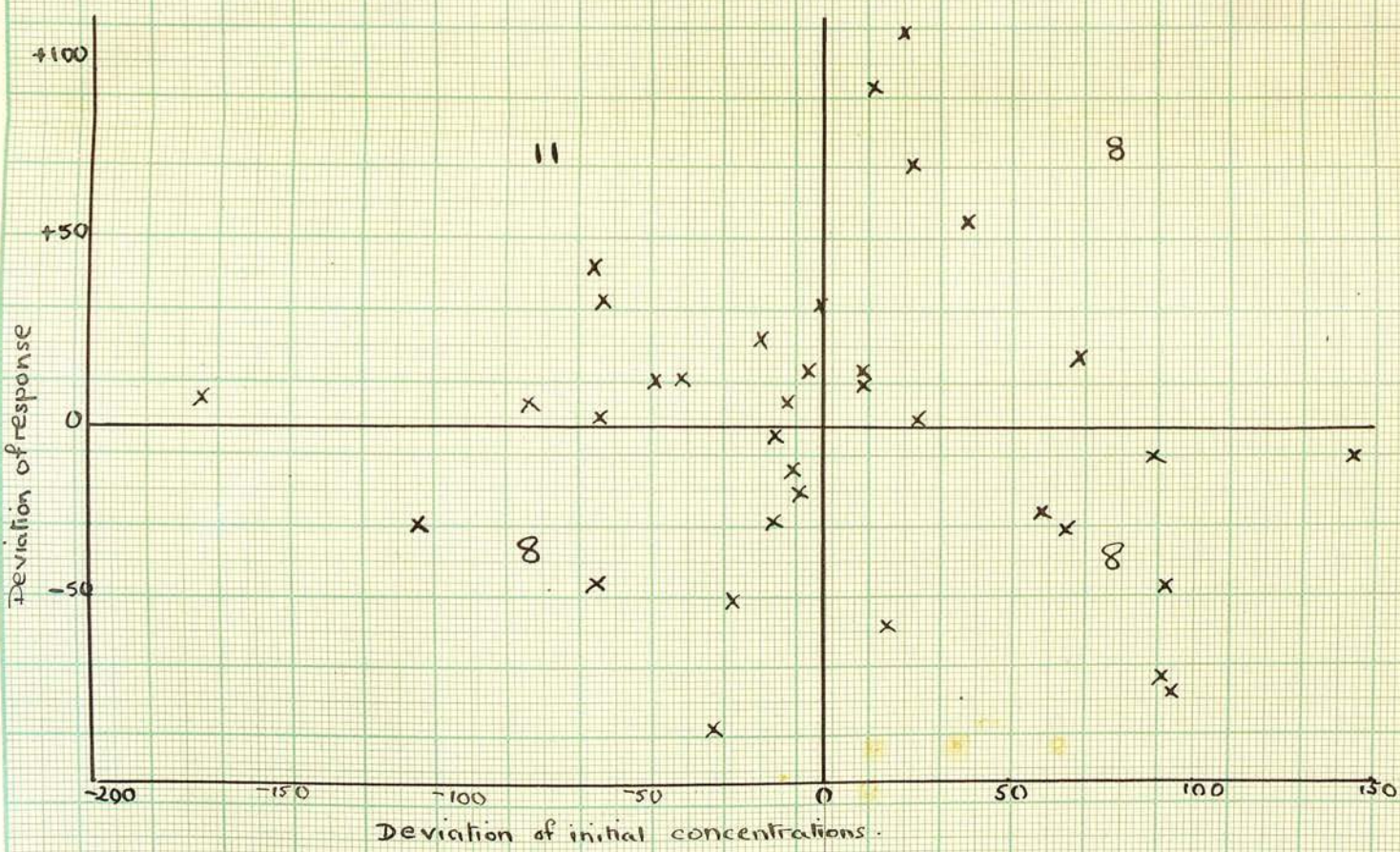


Figure : Effect of deviation of weight and initial concentration on the deviation of response in the Sayers' Test. (compiled by P. Armitage).

The deviation of response against deviation of weight, and the deviation of response against deviation of initial concentration are given in fig. 8.

The precision of the method is illustrated in Table 6. The standard error of the slope is approximately 60, and the standard deviation of 10 slopes is approximately 56. The index of precision, is 0.291 $\pm$ 0.044 compared with 0.176 $\pm$ 0.016 obtained by Sayers et al (1948). The method is thus less precise with this strain of rats, and about $2\frac{1}{2}$ to 3 times as many animals are needed by us to give the same degree of precision as these workers got. Our results compare favourably however with those of Greenspan, Li, Simpson & Evans (1950); they had a slope of 56.6, a standard deviation of 27.9, and λ was 0.499.

Table 6.

Preparation	Number of rats used per preparation.	s	b	λ
Z_3	11	62.7	197.8	0.317
200 : 365	13	38.0	160.3	0.237
203 : 935	11	40.7	128.7	0.316
J.10602	12	71.9	263.2	0.273
British Organon	10	61.3	272.2	0.225
La-1-A	12	57.2	161.4	0.354
J.20507	12	83.6	262.3	0.318

Mean λ = 0.291

Standard error = $\pm$ 0.044

The limits of error usually lie between 50 and 200%, using twelve animals on each of standard and unknown. It was realised that these limits were wide but owing to the small size of our animal colony and shortage of technical assistance, it was considered impracticable to increase the number of animals in order to improve the assays. (The Sayers assays were originally carried out for clinical use, and considered adequate for this purpose.)

Examples of two typical assays obtained by the Sayers technique follow, and the calculations are according to Pugsley (1946).

Table 7. RESPONSES WITH THE INTERNATIONAL STANDARD La-1-A.

The weighed sample was taken up in glacial acetic acid and diluted with saline as already described.

	Body weight in g.	Dose of La-1-A in µg./100g.rat	Weight left adrenal mg.	Weight right adrenal mg.	Mg. ascorbic acid/100g. left adrenal	Mg.ascorbic acid/100g. right adrenal	Depletion	Mean Depletion
1	118	0.6	9.2	9.2	484.6	369.4	-115.2	
2	91	0.6	8.0	5.2	532.4	511.4	-21.0	
3	112	0.6	6.8	5.0	361.6	344.0	-17.6	-93.6
4	82	0.6	6.5	6.8	645.2	470.4	-174.8	
5	166	0.6	7.9	6.6	369.6	230.2	-139.4	
6	110	1.2	8.5	7.0	531.6	485.6	-46.0	
7	131	1.2	6.5	7.3	655.2	391.6	-253.6	
8	133	1.2	9.4	9.1	404.2	241.6	-162.6	-167.3
9	105	1.2	7.0	7.4	494.2	297.2	-197.0	
10	94	2.4	6.9	4.9	434.6	269.4	-165.2	
11	128	2.4	7.4	7.2	540.4	333.2	-207.2	-186.0
12	97	2.4	6.0	6.7	433.2	247.6	-185.6	

s = 57.2

b = 161.4

λ = 0.354

Table 8.

ASSAY OF ARMOUR PREPARATION J.10602.

The preparation was readily soluble in physiological saline.

	Body weight in g.	Dose of J.10602 in mg./100g. rat	Weight left adrenal mg.	Weight right adrenal mg.	Mg.ascorbic acid/100g. left adrenal	Mg.ascorbic acid/100g. right adrenal	Depletion	Mean Depletion
1	110	0.15	8.6	7.4	627.8	467.4	-160.4	-101.8
2	114	0.15	5.3	5.2	792.4	676.8	-115.6	
3	87	0.15	6.4	6.5	675.0	572.0	-103.0	
4	101	0.15	7.7	6.0	571.4	543.2	-28.2	
5	114	0.3	10.3	8.8	580.4	370.0	-210.4	-223.3
6	131	0.3	9.6	7.0	520.8	245.6	-275.2	
7	89	0.3	5.4	6.6	566.6	372.6	-194.0	
8	126	0.3	10.3	10.3	568.8	355.2	-213.6	
9	128	0.6	8.9	7.2	485.4	369.4	-116.0	-260.2
10	90	0.6	6.2	6.1	612.8	371.0	-241.8	
11	104	0.6	6.8	6.7	735.2	346.2	-389.0	
12	98	0.6	6.9	6.6	597.0	303.0	-294.0	
13	101	0.07	7.5	6.3	736.0	771.4	+	No response
14	101	0.07	6.1	5.1	655.6	690.0	+	
15	102	0.07	7.6	5.8	568.4	676.4	+	

$s = 71.9$

$b = 263.2$

$\lambda = 0.273$

$M = \frac{\text{Log. potency of J.10602}}{\text{Potency of La - 1 - A.}} = 0.8042$

With the standard at 100%, J.10602 = 637%

$Sm = 0.1475$

Limits of error (at $p = 0.95$) = 51.4 to 194.6%

Table 9.

ASSAY OF ORGANON PREPARATION 200 : 365

This preparation was taken up in a few drops of glacial acetic acid and diluted in physiological saline as described.

	Body weight in g.	Dose of Organon 200:365 in µg/ 100g. rat.	Weight left adrenal mg.	Weight right adrenal mg.	Mg.ascorbic acid/100g. left adrenal	Mg.ascorbic acid/100g. right adrenal	Depletion	Mean Depletion
1	133	0.6	9.3	8.4	464.4	435.6	-28.8	
2	115	0.6	10.5	7.7	500.8	475.2	-25.6	-42.7
3	81	0.6	7.2	5.2	572.2	500.0	-72.2	
4	113	0.6	9.8	8.8	489.6	445.4	-44.2	
5	94	1.2	7.8	6.7	538.4	456.6	-81.8	
6	121	1.2	9.1	9.0	643.8	480.0	-163.8	-88.7
7	119	1.2	9.4	8.8	531.8	468.0	-63.8	
8	88	1.2	6.7	6.8	486.4	441.0	-45.4	
9	128	2.4	10.4	10.3	505.6	316.4	-189.2	
10	115	2.4	9.2	6.9	469.4	365.2	-104.2	-139.4
11	122	2.4	9.0	6.3	568.8	485.6	-83.2	
12	118	2.4	8.9	8.5	591.0	428.4	-162.6	
13	142	2.4	8.4	7.1	523.8	366.0	-157.8	

s = 38.0

b = 160.3

λ = 0.237

M = $\overline{1}$.6333

With the standard at 100%, Organon 200:365 = 43%

Sm = 0.1024

Limits of error (at p = 0.95) = 62.9 to 158.8%

ASSAY OF ACTH BY ITS EFFECT ON TUMOUR GROWTH.

The possibility of assaying ACTH by its effect on tumour growth was also investigated. Six four - months old male Wistar rats of an inbred strain were given a needle inoculation of tumour T.I.B/5 by Dr. John.

The inoculation was given on 6.4.50. Measurements of tumours were begun on 12.4.50. and ACTH administration to three of the six was begun on 14.4.50. and continued for three days. During that time a total of 69.0 mg. was given to each rat, as three daily subcutaneous injections in saline; controls received saline only. Each morning, the length of the tumour and the diameter of its anterior, posterior and central parts was measured in cms. with a ruler. The following table gives the results:-

Table 10.

Rat	Measurements in cms.	Date 12.4.50.	13.4.50.	Treatment 14.4.50.	15.4.50.	16.4.50.
Saline	Length	5.25	6.0	6.0	7.0	7.25
	Anterior diameter	0.5	1.25	1.5	1.75	2.5
	Mid-point diameter	0.5	1.0	1.0	1.5	2.25
	Posterior diameter	0.5	0.75	1.0	1.5	2.5
Saline	Length	5.0	5.25	5.75	6.5	7.0
	Anterior diameter	0.75	0.75	1.5	1.5	2.5
	Mid-point diameter	0.75	0.75	1.25	1.75	2.75
	Posterior diameter	0.75	1.0	1.5	1.5	2.25
Saline	Length	5.0	5.0	6.0	6.75	7.0
	Anterior diameter	1.0	1.0	1.25	1.75	2.25
	Mid-point diameter	0.5	1.0	1.0	1.5	2.25
	Posterior diameter	0.75	0.75	1.0	1.75	2.75
ACTH	Length	6.0	6.5	6.75	6.75	8.0
	Anterior diameter	0.5	0.75	1.25	1.75	2.25
	Mid-point diameter	0.75	1.0	1.75	1.75	2.0
	Posterior diameter	0.5	1.25	1.25	2.0	2.75
ACTH	Length	5.0	5.25	5.25	6.25	6.5
	Anterior diameter	0.5	0.75	1.0	1.0	2.0
	Mid-point diameter	0.75	0.75	1.25	1.0	2.0
	Posterior diameter	0.5	0.75	1.25	1.5	1.75
ACTH	Length	5.5	5.5	5.75	6.5	7.0
	Anterior diameter	0.5	0.75	1.25	1.5	2.5
	Mid-point diameter	0.25	0.5	1.25	1.5	2.0
	Posterior diameter	0.5	0.75	1.0	1.75	2.0

RESPONSE OF TUMOUR TISSUE TO ACTH.

Thus no clear cut response to ACTH was obtained, and the method was abandoned.

The tumours in the ACTH treated rats had thinner capsules, contained less clotted blood and the tissues surrounding the tumours had a yellowish - green discolouration.

THE USE OF THE WOUND HEALING TEST IN THE EXAMINATION OF OTHER SUBSTANCES FOR A CORTISONE-LIKE ACTION.

1. ɣ - resorcylate.

Reid, Watson, Cochran and Sproull (1951) investigated the antirheumatic action of ɣ - resorcylate and concluded that it was as effective as salicylates in much smaller doses. Through the courtesy of Dr. J. Reid a sample was obtained, and its effect on healing examined. Doses varying from 1.5 to 6 mg. were administered in various ways to pairs of mice and the results obtained are summarised in the following table:-

Table 11.

Dose	Mode of administration	No. of mice	Inhibited	Impaired	Healing
Sodium γ resorcylate					
2 mg.	Solid implanted 6 hours before cutting.	2	0	0	2
4 mg.	As above	2	0	2	0
6 mg.	As above	2	1	0	1
4 mg.	Suspended in arachis oil, and given subcutaneously 6 hours before cutting	2	2	0	0
3 mg.	Aqueous solution given as a series of repeated subcutaneous injections.	2	2	0	0
1½ mg.	As above	2	0	2	0
3 mg.	Aqueous solution as 2 subcutaneous injections 2 hours before and 1 hour after cutting	2	0	0	2
3 mg.	In propylene glycol and saline, as 2 subcutaneous injections, 2 hours before and 1 hour after cutting	2	0	0	2

Controls. With each experiment, controls were cut, and received cortisone diluting fluid, arachis oil, or propylene glycol and saline, as necessary.

RESPONSE OF WOUNDS TO γ - RESORCYLATE.

It has also been given intravenously to hypophysectomised rats in a dose as high as 2.5 mg. per 100 g. rat, but no adrenocorticotrophic activity was detected, the ascorbic acid depletion in three rats being +59.8, +2.4 and +36.2 mg. This does not rule out the possibility of an action on the pituitary gland of the intact animal.

2. Cinchocinic acid.

Through the courtesy of Dr. J. Reid a sample of cinchocinic acid was obtained. Its effect on wound healing was examined.

Dose	Mode of administration	No. of mice	No. of ulcers Inhibited	Impaired	Healing
a) 2.5mg.	Aqueous solution as two subcutaneous injections 1 hour before, and 6 hours after cutting.	3 Male	0	2	1
		2 Female	0	2	0
b) 5.0mg.	Ditto.	5 Male	0	0	5
c) 5.0mg.	Ditto.	2 Female	0	2	0
		2 Male	0	2	0

(With (a) and (c) the solution for the second injection was stored in the refrigerator, and with (b) it was stored at room temperature. This probably accounts for the discrepancies between (b) and (c)). With the doses used, cinchocinic acid was not very effective in the healing test.

3. Dehydroascorbic acid and ascorbic acid.

Long, Miles and Perry (1951 (c)), suggested on experimental grounds (see the discussion later) that dehydroascorbic

acid might alleviate allergic symptoms in rheumatoid arthritis and rheumatic fever, if indeed these are manifestations of bacterial allergy.

If dehydroascorbic acid has in fact a cortisone-like action it would be reasonable to suppose that it could impair healing. Accordingly the effects of dehydroascorbic acid and ascorbic acid were investigated. The sample of dehydroascorbic acid used was the methanolate prepared by Roche, Ltd. and was 80% pure. The doses used and results are given in the following table:-

Dose	administration	No. of mice	No. of ulcers Inhibited	Impaired Healing
Dehydroascorbic acid.				
5 mg.	Aqueous solution as two subcutaneous injections, 1 hour before and 3 hours after cutting.	3	0	0 3
10 mg.	Ditto.	3	0	0 3
Ascorbic acid.				
5 mg.	Ditto.	3	0	0 3
10 mg.	Ditto.	3	0	0 3

Thus, under the conditions of the experiment, no impairment of healing was evident.

EFFECT OF CORTISONE ON HEALING IN GUINEA PIGS.

In view of a report in the literature (Upton & Coon (1951)) that ACTH and cortisone do not inhibit healing in guinea pigs, this problem was investigated.

Granulation tissue was produced by the same technique as in the mouse healing assay. Wounds were left for forty eight hours before examination and profuse granulation tissue was consistently produced in eight control animals.

In preliminary experiments a dosage of 25 mg. cortisone acetate was administered during the period of healing, without effect.

A series of four males, four non-pregnant females and five pregnant females at various stages were given 50 mg. cortisone/day subcutaneously for seven days prior to wounding and 50 mg. each day during the forty eight hours of healing.

At the end of the experiment all animals were healing but macroscopically it was poor in one male and three pregnant females. All ulcers were sectioned and all were in fact healing well, but the layer of polymorphs in the floors of the ulcers considered macroscopically to be poor, was somewhat narrower than in the others, so there may have been less oedema in the treated animals.

No conclusive evidence has therefore been obtained to show that cortisone even in such high dosage can inhibit healing, and the problem remains unsolved.

PHYSIOLOGICAL FACTORS AFFECTING THE RESPONSE OF EXPERIMENTAL WOUND HEALING TO ACTH.

As before, albino mice from our own colony were used and maintained on a diet of Rowett cubes and water. Granulation tissue was produced by the usual technique and a preparation of ACTH, Armour H.7911, was used throughout all these experiments. Pregnant mice, aged six to ten weeks, weighing twelve to eighteen grammes, received 65 µg. or 100 µg. of ACTH during the twenty eight hours of the observation period. Pregnant mice of any weight were used and the dose of ACTH was 500 µg. All operations were performed under ether anaesthesia and all results were confirmed by microscopical examination. Ulcers were fixed and stained as already described. Other organs were fixed in formalin and stained with H. and E.

Effect of Pitressin Tannate and Gonadotrophin on Healing.

There is always a risk that commercial preparations of ACTH may be contaminated by pitressin or gonadotrophin and accordingly it was necessary to investigate their effect on healing. Male mice were given one quarter or one eighth International units of pitressin tannate (Parke Davis, Batch E.319352), dissolved in 0.05 and 0.025 ml. of arachis oil respectively. The dose was divided into two subcutaneous injections given two hours before and six hours after wounding. No evidence of impaired healing or vasoconstriction were seen.

Male and female mice were given 50 International units chorionic gonadotrophin (Boots) dissolved in 0.6 ml. saline as three subcutaneous injections. The first was given when the ulcer was cut and the second and third six and twenty-two hours later. The centres of the floors of the healing ulcers were slightly thinner than usual, but otherwise this excessively large dose of gonadotrophin had no effect on the formation of granulation tissue.

Gonadotrophin Content of the ACTH Preparation used.

The ACTH was tested for luteinizing activity by using it in a four hour rat ovarian hyperaemia pregnancy test, according to the method of Riley, Smith and Browne (1948). The dosage used was 2 mg/rat in 2 ml. saline, and the negative result obtained was as follows:-

Rat No.	Body weight	Injection	Hyperaemia of Right ovary	Left ovary	Uterus
1	45 g.	ACTH	-	-	-
2	59 g.	ACTH	-	-	-
3	54 g.	ACTH	-	-	-
4	58 g.	Saline	-	-	-

It was also assayed for follicle stimulating hormone activity by the method of Klinefelter, Albright & Griswold (1943). Each mouse received a total of 2.5 mg. ACTH during the test period, and no effect on uterus or ovaries was produced. Thus, no evidence of contamination by gonadotrophin was obtained.

Operative Interference with the Mice, and its Effect on Healing.

Using groups of eight or more mice, we found that any operative interference produced some impairment of healing. This was least marked in males and females after splenectomy or gonadectomy, more so after hypophysectomy, and most after adrenalectomy. As the time between operative interference and making the ulcer is increased, so does the impairment become less. In all instances, therefore, definite healing occurs, the degree varying according to the type of operation carried out and the time elapsing before making the ulcer. By carefully controlling all experiments this has not proved a disadvantage.

Effect of Hypophysectomy.

Hypophysectomy was performed by the parapharyngeal route, using light ether anaesthesia and the technique already described for rats. There is more tendency to bleed in mice, but it is readily controlled with spirit. The effect of ACTH varied according to the time between hypophysectomy and making the ulcer, in both sexes. The results are shown in Table 12.

Table 12.

Time between hypophysectomy and making the ulcer (days).	No. of mice in group	Treatment	Effect on healing		
			Complete inhibition	Impaired	Normal healing
1	4	Saline	0	0	4
	4	ACTH	3	1	0
2	4	Saline	0	0	4
	3	ACTH	0	2	1
3	5	Saline	0	0	5
	3	ACTH	0	0	3

Thus hypophysectomized mice show inhibition when only one day is allowed to elapse between hypophysectomy and wounding; the power to respond is soon lost.

Effect of Gonadectomy.

ACTH administered after gonadectomy of male and female mice failed to produce any inhibition of healing. Mice under going adrenalectomy plus gonadectomy showed poor but definite healing which was not inhibited by ACTH. The survival rate of these animals was, however, very poor and about one in six recovered. Detailed results are shown in Table 13 . Ovaries were removed by a dorsal approach and testes were removed through the wall of the scrotum. Healing advanced more rapidly in ovariectomised females than in intact females so that at twenty eight hours healing resembled that seen in intact males.

TABLE 13.

EFFECT OF ACTH ON WOUND HEALING IN GONADECTOMIZED, AND GONADECTOMIZED PLUS ADRENALECTOMIZED MICE.

Operation	Time between gonadectomy and wounding (days)	No. of mice in group.	Treatment	Completely Inhibited	Impaired	Normal healing
Ovariectomy	2	16	Saline	0	0	16
		10	ACTH	0	2	8
Ovariectomy	1	4	Saline	0	0	4
		4	ACTH	0	0	4
Ovariectomy and adrenalectomy	2	1	Saline	0	0	1
		2	ACTH	0	0	2
Removal of testes	2	7	Saline	0	0	7
		10	ACTH	0	0	10
	1	4	Saline	Variable and poor healing in all animals;		
		4	ACTH	no difference between the two groups.		
Removal of testes and adrenals	2	4	Saline	0	0	4
		2	ACTH	0	0	2

Effect of Adrenalectomy.

The adrenal glands were removed through the dorsum. Healing was studied one, two, six and eight days after adrenalectomy in groups of four mice. At one day healing was almost non-existent but at two, six and eight days after adrenalectomy healing was evident though the wounds had a more yellow appearance than in intact animals and the floors were thinner than usual. Since two days gave reasonable healing, but little time in which any extra-adrenal tissue might hypertrophy, this interval was allowed to elapse between adrenalectomy and making the ulcer in subsequent experiments. The mortality amongst these animals was very high once the dressing was applied. They had to be kept warm, and given glucose-saline from a pipette at frequent intervals. Similar results have been obtained in male and female mice (see Table 14).

Table 14.

EFFECT OF ACTH ON WOUND HEALING IN ADRENALECTOMIZED MICE.

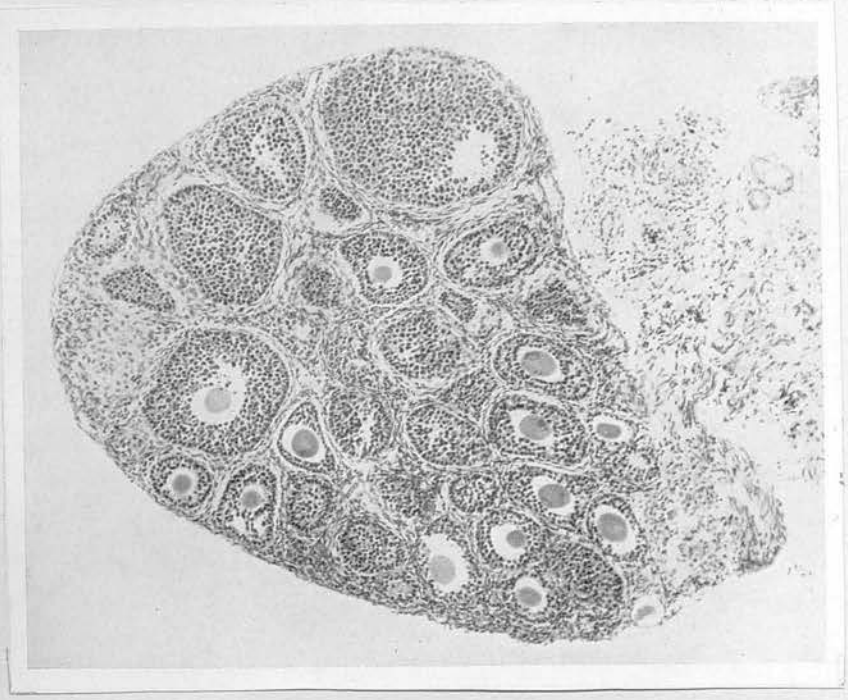
Date	No. of mice in group	Treatment	Effect on healing: Completely inhibited	Impaired	Normal healing
Second half November 1950	6	Saline	0	0	6
	8	ACTH	7	1	0
First half December 1950	4	Saline	0	0	4
	4	ACTH	1	3	0
First half of January 1951	8	Saline	0	0	8
	9	ACTH	5	0	4
Second half of January 1951	6	Saline	0	0	6
	13	ACTH	0	1	12
Second half of February 1951	2	Saline	0	0	2
	4	ACTH	0	1	3
Second half of April 1951	6	Saline	0	0	6
	7	ACTH	0	0	7

During the second half of November 1950 it was found that even though the adrenals were absent, ACTH still produced inhibition. In December 1950 and the first half of January 1951, only some adrenalectomized mice showed inhibition, and by the second half of January none showed complete inhibition.

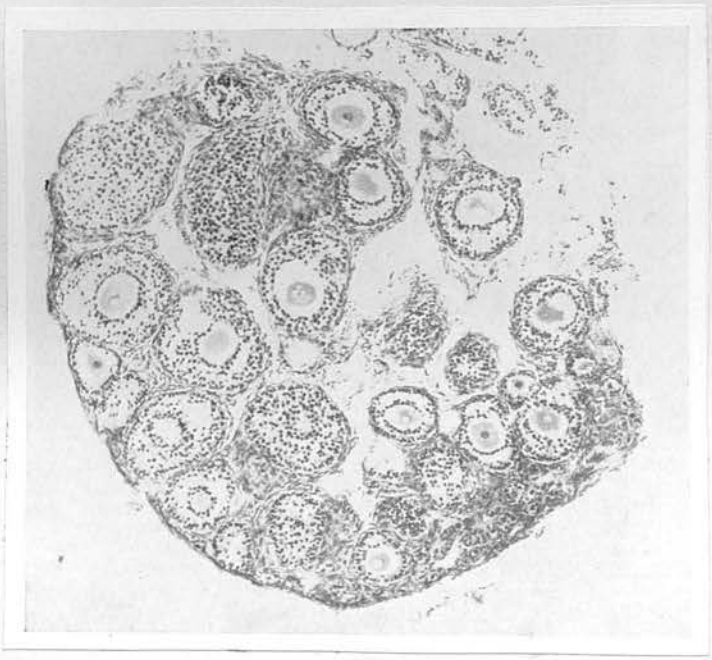
It seemed possible that a seasonal change might have been taking place, so that in November and December the presence of the gonads alone was sufficient to cause inhibition in response to ACTH, but that later in January the adrenals were also essential. The changes observed can be correlated with another possible seasonal change in the same colony.

Since October 1949, the colony has been used to estimate urinary FSH excretion by the method of Klinefelter et al. (1943). This depends on the ability of extracted urinary FSH to cause indirectly a large increase in the size of mouse uteri. The twenty-one day old uteri have consistently weighed 2-3 mg. except for an interval in the early part of the winter, 1949 - 1950, when they weighed 7010 mg. and occasionally even more. On sectioning, such uteri showed a larger surface area of epithelium than usual, and the endometrium contained more connective tissue. The ovaries of such animals showed developing follicles in which there was a suggestion of proliferation of the granulose cells and antrum formation (fig. 9). During this time, too, the uteri are unduly but irregularly responsive to FSH.

After a lull in breeding during September and early October these mice were the first offspring produced during the



(a)



(b)

Figure 9.

(a) Normal 21 - day old mouse ovary x 42.

(b) Ovary from a mouse 21 days old at the end of November, 1949, stained with haematoxylin and eosin x 42.

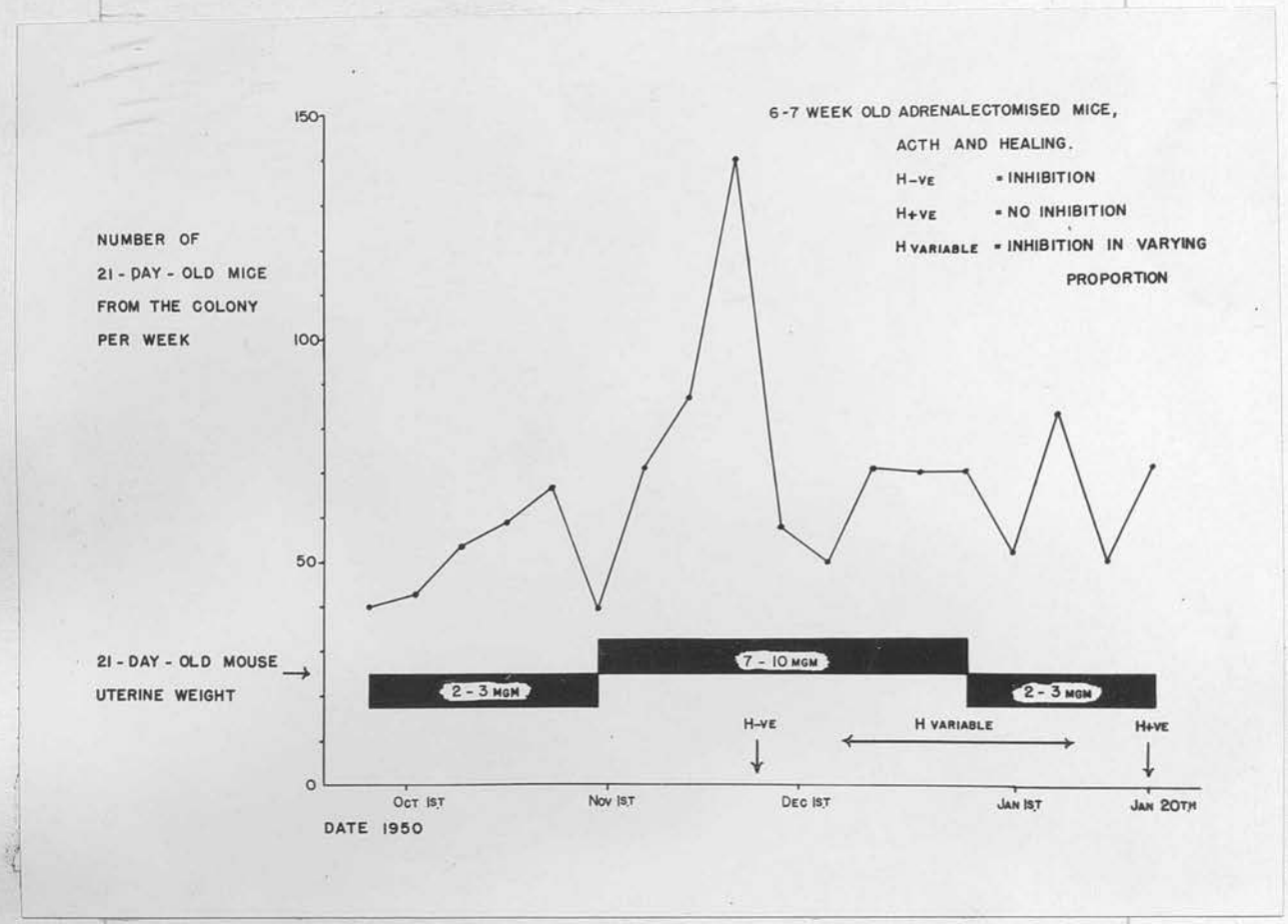


Figure 10. Healing in 6 - 7 week old adrenalectomised mice at the time indicated.

new breeding season. The twenty-one day old mice maturing prematurely were litter-mates of the six to nine weeks old adrenalectomized mice in which ACTH could cause inhibition of healing. The correlation between the number of twenty-one day old offspring produced by the colony, uterine size, and response of adrenalectomized mice to ACTH is shown in fig. 10.

Effect of Pregnancy on the Response of Adrenalectomized Mice.

Ulcers were made on four mice in the third week of pregnancy, and the usual vaseline gauze and cap dressing was supplied. In all instances the dressings slipped and the wounds became contaminated. This probably arose from the abdominal contour, and the difficulty of applying the dressing firmly.

Other dressings were, therefore, tried on single mice:-

(a) The wound was covered with collodion only.

(b) The wound was smeared with vaseline and then covered with collodion.

(c) The wound was covered with vaseline gauze. Over this was placed a piece of cellophane paper and the whole was covered with collodion.

(d) The wound was covered with vaseline gauze. A small ball of cotton wool was placed over the gauze and held in place with loosely applied adhesive tape.

It was found that:-

(a) and (b) were unsuccessful.

(c) remained in place but was movable and tore the granulations.

(d) was successful; it remained in situ and granulations formed underneath. Accordingly this dressing was adopted routinely in pregnant mice.

Mice were adrenalectomised at various stages of pregnancy and immediately post partum. Ulcers were cut two days later. Some of the mice aborted after adrenalectomy and some after the ulcers were made. Others remained pregnant until the end of the experiment.

Six mice from the first, second and third weeks of pregnancy, and two immediately postpartum were adrenalectomized and ulcers were made. They received saline injections and all showed good healing, though in the last week of pregnancy capillary formation was poorer.

A series of nine mice received ACTH and gave the following results:-

State of pregnancy	Effect of ACTH on ulcer
1. The mouse remained pregnant. On killing, 7 well-advanced dead foetuses were found in the uterus.	Complete inhibition
2. The mouse remained pregnant. On killing 9 living well-advanced foetuses were found in the uterus.	Complete inhibition
3. The mouse remained pregnant. On killing, 8 foetuses at approximately mid term were present (probably dead).	Partial inhibition
4. The litter was born about 6 hours before killing.	Complete inhibition.
5. The litter was born during the first few hours after cutting.	Complete inhibition.
6.} The litter was born the day 7.} following adrenalectomy.	Partial inhibition.
8.} Adrenalectomy was performed 9.} one day postpartum. The young were left with the mothers, and the dressing was applied so that lactation could continue.	Both healed well

The placentae and foetuses showed no abnormality on naked-eye examination.

In these experiments about one quarter of the pregnant mice died before the experiment was finished, and were discarded.

Effect of Pre-treatment of Non-Pregnant Mice with Gonadotrophins.

1. Pregnant mares' serum gonadotrophin.

A series of female mice, aged six weeks, were treated with pregnant mares' serum (Boots), 2.5 I.U. twice a day subcutaneously in 0.1 ml. saline for ten days. They were then adrenalectomized, and two days later the ulcers were made. Gonadotrophin administration was continued during this period. Three were given saline and six received ACTH. Healing was variable and rather poor, and no significant differences could be detected between the two groups. Adrenalectomized mice, pre-treated with PMS did not respond to ACTH.

2. Post-menopausal follicle-stimulating hormone.

After pre-treatment with crude FSH extracted from 60 ml. post-menopausal urine, by kaolin adsorption and acetone precipitation (Loraine, (1950)), and administered as two subcutaneous injections daily for ten days, a series of female mice were adrenalectomized and two days later the ulcers were made. Healing was poor in three treated with saline and in three treated with ACTH, and there was no significant difference between the groups.

3. Chorionic gonadotrophin.

An experiment similar to that with PMS was carried out

in female mice with chorionic gonadotrophin (Pregnyl). Each mouse received 2.5 I.U. twice daily subcutaneously in 0.1 ml. saline for ten days. Nine adrenalectomized controls treated with saline showed moderate healing. Eight which received ACTH showed varying degrees of inhibition. The wounds of seven of these were pale with very steep margins, smooth floors, and no hyperaemia - i.e. typical inhibition by ACTH. One was hyperaemic though its margins were swollen and the floor was smooth. It was interesting that this mouse had the smallest uterus, weighing only 2.5 mg. The results in this small series suggested that the greater the uterine sizes, the more severe was the impairment of healing by ACTH. The mean control uterine weight was 15.2 $\pm$ 3.9 mg. and the mean treated uterine weight was 52.2 $\pm$ 8.7 mg. The ovaries of the pre-treated mice showed extensive luteinization, and after ACTH treatment the ovaries were more hyperaemic and contained some haemorrhagic follicles.

4. Survival of animals in the tests with gonadotrophin.

In each of the three experiments, twenty mice were injected with gonadotrophin. Following adrenalectomy or making the ulcers, a number died and the ultimate survivals were:-

(a) with pregnant mares' serum gonadotrophin - 9

(b) with post-menopausal follicle stimulating hormone - 6

(c) with chorionic gonadotrophin - 17

The survival with chorionic gonadotrophin, therefore, was much higher, and these animals were strikingly healthy at the end of the experiment compared with those of the other two groups.

5. Chorionic gonadotrophin in male mice.

The experiment with pregnyl was repeated using male mice. Of a group of twenty, seven survived the complete operation and even then were unhealthy. Two treated with saline showed rather poor healing; of five which received ACTH, three showed very little healing though inhibition was never complete. The testes were weighed, and impairment of healing was more marked in those with the larger testes, in this very small series.

Treatment	Body weight in g.	Weight of two testes in mg.	Healing
Untreated controls	13.5	93	
	11.0	86	
	16.5	86	
	17.5	118	
	12.0	76	
Pregnyl and saline	11.5	82	Fair
	13.0	60	Fair
Pregnyl and ACTH	13.5	104	Poor
	15.0	102	Very poor
	15.0	122	Very poor
	12.0	81	Fair
	14.0	70	Fair

The adrenal glands of the female mice treated with pregnyl showed a zone of hyperaemia at the cortico-medullary junction. The cortex was not thickened but there was more lipoid in the outer part of the zone fasciculata and zona glomerulosa.

An attempt has been made to detect ovarian changes as a result of ACTH administration to C.G. treated adrenalectomized mice. Untreated mice had small pale ovaries and very thin uteri. Following C.G. treatment, the uteri became thick and fleshy, some follicles showed early luteinisation, and an occasional haemorrhagic follicle was seen. Following adrenalectomy, haemorrhagic follicles occurred more frequently. Following ACTH, haemorrhagic follicles were numerous, particularly after four hours treatment with ACTH and less after twelve and twenty four hours. Those at twenty four hours were of varying shades of red and some looked very dark. At four hours especially, all the abdominal vessels were very dilated; an occasional animal had blood - stained intraperitoneal fluid and an occasional ovary was surrounded by a clear cyst containing fluid which was sometimes blood - stained. The reproductive organs were fixed in formalin and stained with H. and E. after sectioning.

With the assistance of Dr. J. Bamforth the sections were carefully examined, but no cell types which might account for the action of ACTH in inhibiting healing in the absence of adrenals, were observed at all.

Effect of some Steroids on Healing.

Since gonads which have been under the influence of luteinizing hormone have shown a response to ACTH, the effect of some steroids on healing has been investigated. The results obtained with cortisone acetate, oestriol, oestradiol benzoate, testosterone and progesterone are shown in Table Control mice received saline, propylene glycol and saline or 'diluting fluid' (supplied by the manufacturers) as necessary. Cortisone gave complete inhibition in both intact and gonadectomized mice. Of the other steroids used, only progesterone gave some definite impairment under the conditions of the test and in the dosages employed. This impairment was not nearly so definite as that produced by ACTH.

Table 15.

EFFECT OF STEROID HORMONES ON WOUND HEALING

Steroid	Dose mg.	Mode of injection	Type of mouse	No. in group	Effect on healing
Cortisone acetate	4	Merck's suspension 2 hr. before making the ulcer.	Infact males and females	11	Complete inhibition in all
	4	Merck's suspension 2 hr. before making the ulcer.	Gonadectomized males and females	7	Complete inhibition in all
	2	Suspension in propylene glycol and saline, intraperitoneally 2 hr. before wounding.	Intact males and females	4	Complete inhibition in all
Oestriol	2.5	In saline, subcutaneously, as two injections, 2 hr. before and 4 hr. after wounding.	Intact males	2	No effect on healing
			Intact females	2	No effect on healing
Oestradiol benzoate	2.5	In 'diluting fluid' as two subcutaneous injections, 2 hr. before and 4 hr. after wounding	Intact males	2	No effect on healing
			Intact females	2	No effect on healing
Testosterone	2.5	Suspension in propylene glycol and saline, as two subcutaneous injections, 2 hr. before and 4 hr. after wounding	Intact males	2	Possibly slight impairment
			Intact females	2	No effect on healing
Progesterone	2.5	In 'diluting fluid' as two subcutaneous injections, 2 hr. before and 4 hr. after wounding.	Intact males	4	By naked eye examination slight impairment was found in the females; it was more marked in the males. Microscopically, all showed some impairment but sex distinction was not evident.
			Intact females	4	

Effect of ACTH on Adrenalectomized Mice.

There was always a risk that extra-adrenal tissue might be present, in spite of careful examination of the animals. Accordingly, attempts were made to hypertrophy any extra-adrenal tissue by the administration of ACTH to adrenalectomized mice. ACTH was administered to a small series of three week old adrenalectomized male and female mice in February 1951, in a dosage of 45 µg./day as two injections daily for ten days. Ulcers were then made, half the mice received saline and the other half repeated injections of ACTH. Results showed that healing was poor and irregular in both groups, and no greater impairment occurred in those which had received ACTH during healing, thus ruling out the presence of extra-adrenal tissue. It was interesting to note that whereas untreated adrenalectomized mice lost or failed to gain weight, those mice receiving daily ACTH usually gained weight, and in addition the scrota, vulvae, and dependent parts of their faces were swollen and oedematous. The weight changes are shown in Table 16.

Table 16.

Adrenalectomised mouse	Treatment	Weight at time of adrenalectomy. In g.	Weight at end of experiment in g.
1. Male	Saline	16.0	14.5
		16.0	16.5
		17.0	16.5
2. Male	ACTH	16.5	18.5
		14.6	19.0
		13.0	15.0
		13.0	18.5
3. Female	Saline	16.0	15.5
		14.6	12.0
		18.0	18.0
4. Female	ACTH	17.5	19.0
		17.5	19.5
		13.0	11.5

Healing in Mice 1 - 7 days old.

In view of the part played by the gonads, healing was investigated in mice, aged 1 - 7 days. The wounds were dressed with vaseline gauze and a ball of cotton wool, and the dressing was kept in place with adhesive tape. The young were left with their mothers who were also smeared with vaseline. At this age, they weighed 2.7 to 3.5 g. Healing was examined in three females and three males after twenty eight hours; the floors were thickened and capillary formation had begun in the females, but was less advanced in the males (a reversal of the effect seen in mature mice). A further two females and five males were examined after thirty six hours, and healing was more advanced in them all, and the sex distinction was not so apparent.

Unsuccessful attempts were made to inhibit healing with 45 µg. ACTH H.7911, five females and four males were treated. Macroscopically I thought healing was grossly impaired, but on sectioning there was profuse polymorph infiltration though no capillary formation, so the gonads appeared to be playing a part even in the very young animals.

Attempts to adrenalectomize 1 - 7 day old mice were successful, but they could not withstand the making of the ulcer, and the experiment had to be abandoned.

The slower increased healing in the males is interesting, as in the adult mice, the males heal more rapidly.

ADRENAL ASCORBIC ACID IN MICE.

As a point of interest, the adrenal ascorbic acid was determined in a series of mice by the dinitrophenylhydrazine method. Pooled groups of two to eight glands were used.

The results were as follows:-

Table 17.

Description of mice.	Adrenal ascorbic acid in mg/100 g. using pooled groups.	Mean adrenal ascorbic acid in mg/100g.
Intact males	310.0 212.0 430.0 282.0 484.8 314.2	338.8
Intact females	190.0 237.0 392.5 232.8 230.6	256.6
Males treated with oestrogen	129.4 163.5	146.4
Females treated with oestrogen	162.2 139.8	151.0
Pregnant females	154.0 275.7 214.2 212.6	214.1
Postpartum females	154.8	154.8
Females treated with pregnant mares' serum gonadotrophin	433.7 296.4 292.3	340.8
Females treated with postmenopausal follicle stimulating hormone	236.1 279.0	257.5
Females treated with chorionic gonadotrophin	117.0 154.0	135.5

The numbers are small, but it does appear as though oestrogen and chorionic gonadotrophin depress the adrenal ascorbic acid, while P.M.S. gonadotrophin and F.S.H. gonadotrophin do not.

EFFECT OF CHORIONIC GONADOTROPHIN AND ACTH ON THE ASCORBIC ACID OF TESTES.

In view of the mouse healing results it was thought that it would be interesting to investigate the effect of chorionic gonadotrophin and ACTH on the ascorbic acid content of testes. The experiment was performed on intact male mice weighing about 30 g. The left testis was removed under light ether anaesthesia and a subcutaneous injection of 10 I.U. chorionic gonadotrophin (C.G) or 50 mg. ACTH H.7911 in saline was given subcutaneously in the back of the neck. The right testis was removed one or four hours later. The testes were placed in trichloracetic acid and ascorbic acid determined as usual.

The results were as follows:-

Table 18.

Injection	Time between adrenalectomies in hrs.	Ascorbic acid content of testes in mg/100 g.	
		Left	Right
Saline	1	61.8	58.7
"	1	51.3	46.6
"	4	42.6	42.6
"	4	52.9	59.3
10 I.U. C.G.	1	52.3	54.4
"	1	40.7	51.8
"	1	45.2	45.7
"	4	47.5	56.6
"	4	47.2	47.0
"	4	45.4	46.9
ACTH	4	51.2	47.7
"	4	60.7	61.6
"	4	55.8	49.1
"	4	60.0	63.0

No evidence of depletion as a result of C.G. or ACTH was found, and indeed the ascorbic acid concentrations were extremely uniform.

PART II

ADRENAL FUNCTION IN GUINEA PIGS, WITH PARTICULAR REFERENCE TO SCURVY.

INTRODUCTION II

Within recent years there has been much interest in the role of ascorbic acid in adrenocortical function, though the problem has remained essentially unsolved.

It has been known for many years that the adrenal gland contains a very high concentration of ascorbic acid (Szent-Gyorgi (1928)) and more recently the increasing amount of information on adrenal steroids has centred interest on the relationship between ascorbic acid and adrenal steroid metabolism. Following the work of Sayers, Sayers, Lewis & Long (1944) and Sayers, Sayers, Liang & Long (1946) it has become well-known that pituitary adrenocorticotropic hormone which stimulates the release of adrenal steroids, also causes a fall in adrenal ascorbic acid. This is true for the rat, which can synthesise ascorbic acid, and for the guinea pig which is unable to do so (Sayers et al (1946), Long (1947a, 1947b)), and therefore is dependant upon an adequate dietary intake. In the absence of ascorbic acid, the guinea pig develops scurvy with all its well-known manifestations (Bessey, Menten & King (1934)).

It seemed profitable to examine adrenal function in scurvy when the ascorbic acid content was known to be very low, and it was with some surprise that a rise in urinary 17-ketosteroids was consistently observed as acute scurvy developed or as a terminal manifestation in chronic scurvy. Experiments have also shown that the adrenal gland is still capable of responding to exogenous ACTH when ascorbic acid is

minimal. Until recently there has been no other work along these lines.

It is usually considered that urinary 17-ketosteroids arise from the adrenal cortex, and to a lesser extent from the testes in the male (Fraser, Forbes, Albright, Sulkowitch and Reifenstein (1941)). Experiments have in fact demonstrated that the increased 17-ketosteroids are of adrenal origin, and that the gonads are not contributing to it to any significant extent.

Observations have been made on the effect of cortisone and ACTH on scorbutic guinea pigs with particular reference to the development of symptoms and signs and the excretion of 17-ketosteroids. From 1933 to 1944 there occurred a series of papers on the influence of cortical extracts on the course of scurvy. Lockwood & Hartman (1933), Lockwood & Hartman & Hartman (1933) and Ratsimamanga (1944), claimed that it had a beneficial effect while Vars & Pfiffner (1934), Grollman & Firor (1934) and Svirbely & Kendall (1936) found that it exerted no influence at all. The reason for these discrepancies was not apparent, though the argument was put forward that some extracts might have contained ascorbic acid. While the experiments described in this thesis have been in progress, a number of papers have been published along similar lines and these are described in the "Discussion"; the results are very conflicting.

In 1950, Schaffenburg, Masson & Corcoran (1950) reported a very interesting experiment, in which they claimed that cortisone exerted a beneficial effect in scurvy. They divided twenty six male guinea pigs, weighing 280 - 300 g. into four groups, and fed them on the scorbutic diet of Schultz (1936). Group I received 5 mg. DOCA daily. Group II were given 5 mg. cortisone/day by injection, increased to 7.5 mg. on the eleventh day and to 10 mg. on the nineteenth day. Group III received 5 cc. orange juice daily and Group IV were untreated. In addition Groups I, II and IV received 1 cc. orange juice on the 4th, 8th and 11th, days to favour the development of chronic scurvy. Groups I and IV lost weight at the same rate after the tenth day, but II and III gained weight at the same rate until the end of the experiment when Group II barely maintained it (see fig. 11). Thus, as a result of cortisone administration, a group of animals on a scorbutic diet were enabled to gain weight at the same rate as animals on a diet supplemented with adequate amounts of orange juice. Unfortunately they terminated their experiment at 21 days. They also claimed that cortisone prevented enlargement of the joints in all except one animal, and reduced the haemorrhagic manifestations. The weight curves given in their paper have been copied in fig. 11 and it will be seen that the effect is indeed striking. It was decided that an attempt should be made to repeat this work with some modifications. The experiment was carried on until the animals died, and ascorbic acid was used as a supplement instead of

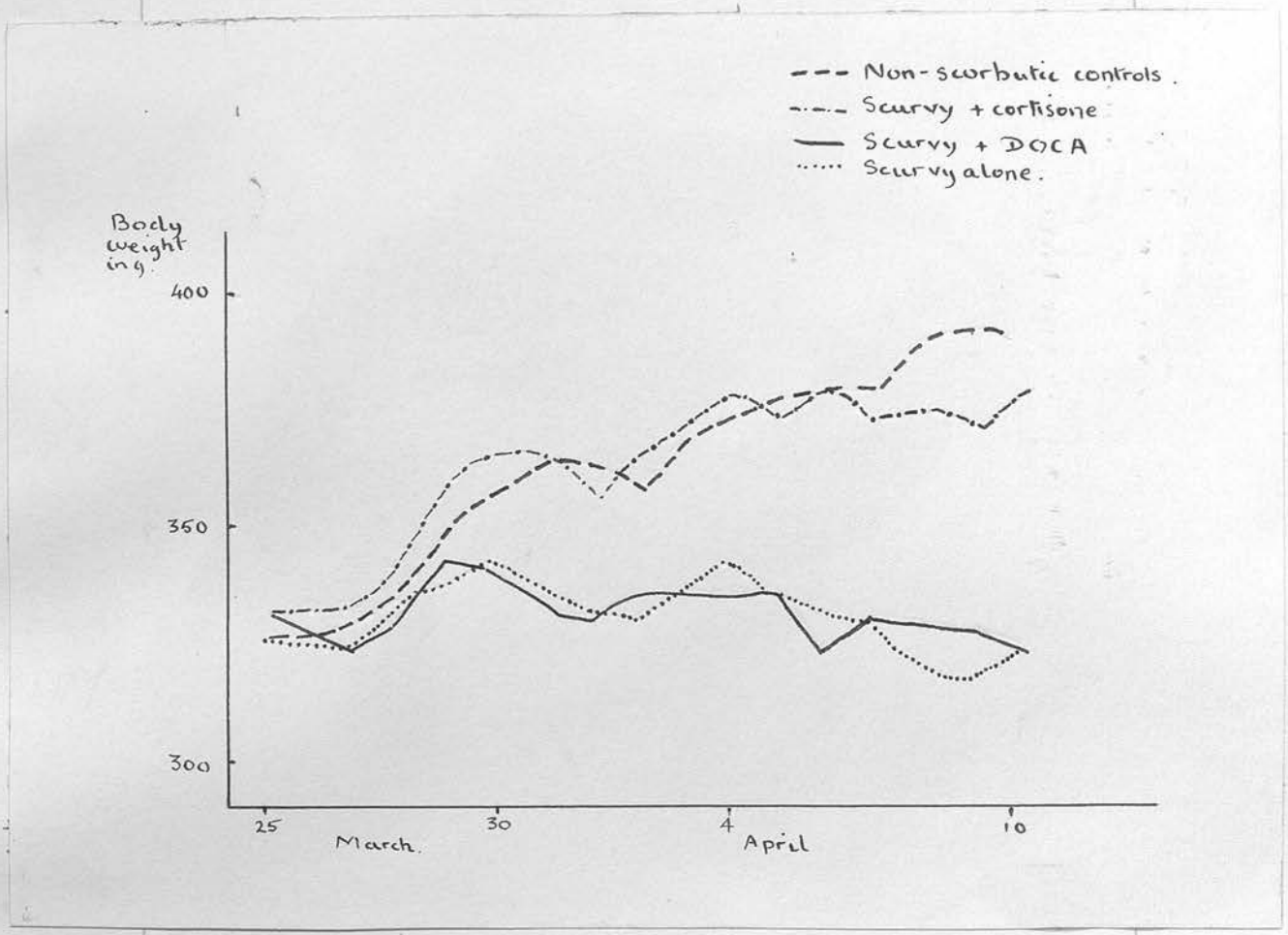


Figure 11. Reproduced from the paper of Schaffenburg, Masson & Corcoran (1950). Effect of cortisone on the growth curve in scurvy.

orange juice, Since this experiment failed to confirm that of Schaffenburg et al (1950) it was repeated using cabbage as a supplement, but again no confirmation was obtained. The experiments are reported in detail in the experimental part of this thesis.

While the experiments reported in this thesis were in progress, Gallagher, Fukushima, Barry & Dobriner (1951) described an experiment in which steroids were found in faeces. They studied the neutral ketonic fraction of the faeces of normal men and failed to demonstrate any known keto-steroid. They then injected radioactive testosterone labelled at carbon - 4 intraperitoneally and recovered 30 - 40% of it in the urine and about 60% in the faeces within the first 24 hours. In rats, the percentage recovery was somewhat less and almost all of it was recovered from the gastro-intestinal tract or faeces. It therefore seemed possible that significant amounts of 17-ketosteroids might be present in guinea pig faeces, particularly during ACTH or cortisone treatment. Experiments have therefore been carried out to examine this point.

The colour produced by ketosteroids other than 17-keto-steroids in the Zimmermann reaction has been examined by Callow, Callow & Emmens (1938). Δ^4 - 3 - ketones showed a moderately intense absorption throughout from 450 to 650 mμ, with peaks at about 450 and 570 mμ. Saturated 3 - and 20 - ketones showed a low general absorption over the same range, without marked peaks, but more intense at the lower wave-lengths than at the higher.

Long, Miles & Perry (1951 abc) have published a very interesting series of observations.

They found that ascorbic acid substantially desensitized guinea pigs made sensitive to tuberculin by B.C.G. vaccine. This effect was inhibited by a cabbage factor, since it did not occur in their animals which received cabbage as a source of ascorbic acid. On the other hand, ACTH and cortisone desensitize and their action depends on a reversal of the cabbage effect. Accordingly they have studied the first oxidation product of ascorbic acid i.e. dehydroascorbic acid (DHA). It was found that DHA densensitizes in the presence or absence of cabbage, so they suggested that the cabbage factor might inhibit desensitization by ascorbic acid by preventing the oxidation of ascorbic acid to DHA. They also showed that injection of the sulph hydryl compound, glutathione, inhibited ascorbic acid densensitization and so suggested that the cabbage factor might be an -SH compound. Accordingly they thought it possible that DHA might alleviate allergic symptoms without producing disturbing side effects. If indeed rheumatic fever and rheumatoid arthritis were manifestations of bacterial allergy, then they thought it possible that DHA might produce relief.

An investigation of the physiological effects of DHA in animals was necessary prior to its use in human patients.

A certain amount of work had already been done on this by Patterson, (1949, 1950) and by Patterson & Lazarow (1950).

He prepared his DHA by the oxidation of ascorbic acid with quinone, and found that if injected into rats it could lead to the development of permanent diabetes. Immediately after an intravenous injection they became excited, ran round their cages and gasped for breath. The prior administration of a sulph hydryl compound protected them. Patterson suggested that DHA might interfere with the essential sulph hydryl enzymes of the β cells of the Islets of Langerhans. DHA has been given to rats by the writer without obtaining any striking effects at all. They exhibited a slightly gasping respiration shortly afterwards for a few minutes but that was all, and it was considered that it could be merely an acidosis effect.

An antiscorbutic effect has been claimed for DHA by Borsook, Davenport, Jeffreys & Warner (1937) who made it by the oxidation of ascorbic acid with iodine, and by Gould & Shwachman (1943) using a similar preparation. The report of Roe & Barnum (1936) claiming little antiscorbutic activity may have been due to a chemical error.

EXPERIMENTAL II

ADRENAL FUNCTION IN SCURVY.

EXPERIMENTAL

17-KETOSTEROID EXCRETION DURING UNTREATED ACUTE SCURVY.

Methods.

Male and female guinea-pigs of varying weights (300 to 590 g.) obtained from two registered breeders were kept in groups of two or three in metabolism cages. They were maintained on the scorbutic diet described by Harris and Ray (1932), which was supplemented by cabbage ad lib. where healthy pigs were required. Tap-water and the scorbutic diet were supplied ad lib. and in some experiments the amount eaten each day was measured. Body weights were determined daily.

Daily, 24-hour specimens of urine were collected over concentrated A.R. hydrochloric acid, except on Sundays. Urinary 17-ketosteroids were determined at frequent intervals. Of each group of animals one was killed when moribund and the organs were sectioned. All animals were examined at death, and the diagnosis of scurvy was confirmed. In some healthy and scorbutic animals the ascorbic acid content of the adrenal glands was determined by the method of Roe and Kuether (1943).

Determination of 17-ketosteroids. Acid hydrolysis was performed by gentle boiling for ten minutes with 15 mls. concentrated hydrochloric acid per 100 mls. urine in a conical flask without a reflux condenser. The urine was cooled rapidly under a tap and extracted four times with 50 ml. carbon

tetrachloride. The extract was then evaporated under vacuum at 50°C, and the distillation head was washed with peroxide - free ether. The extract was taken up in 80 mls. ether and washed once with 10 ml. distilled water, twice with 10 mls. saturated sodium carbonate solution, four times with 10 ml. 2 N caustic soda solution and twice with 10 ml. distilled water. The last washing was left to stand for one hour. The ether was then evaporated to dryness at 50°C. The Zimmermann reaction as described by Callow, Callow & Emmens (1938) was carried out and a colour correction was finally applied according to Talbot, Berman & MacLachlan (1942).

Results.

In normal male and female guinea-pigs the daily 17-ketosteroid excretion has varied between 0.09 and 0.30 mg/24 hours.

In Table 19 a series of results is given for four separate groups of animals. This shows the daily average body weight in grammes, and the average 17-ketosteroid excretion in milligrams per 24 hours. In addition, for group I, details of daily food and daily urine volume are given. In Table 20 the day on which a fall in body weight began and the day of death are given for 13 animals.

Table 14.

17-Ketosteroid excretion during scurvy.

		Female Group I Experiment I (2 pigs)				Male Group II. Exp.II (3 pigs)		Female Group III. Exp.III (2 pigs)		Male Group IV. Exp. IV (2 pigs)	
Diet	Day of experiment	Average body weight in g.	Average food intake in g/24hrs.	Average urine volume in cc/ 24 hours	Average 17-ketosteroid excretion in mg/24hrs.	Average body weight in g.	Average 17-ketosteroid excretion in mg/24hr.	Average body weight	Average 17-ketosteroid excretion in in mg/24hrs.	Average body weight in g.	Average 17-ketosteroid excretion in mg/24hrs.
Scorbutic diet	1					485.0	0.200				
+cabbage ad lib.	2	521.5				450.0	0.100	302.5	0.119	350.0	0.098
Scorbutic diet.	1	521.5				451.0	0.300	281.5	0.076	336.5	
	2		24			442.0		337.0		326.0	
	3	455.0	24	12.5	0.176	437.0		347.5		364.0	
	4	439.5	23	10		435.5	0.300	357.0		382.0	0.198
	5	465.0	43	7		434.0		321.5			
	6	496.0	45	12	0.119	426.5					
	7	487.5	42	16				340.0		377.5	
	8	494.0	43	11	0.204	426.0	0.360	353.5	0.187	380.5	0.148
	9		27	12		421.5		358.0		396.5	
	10	484.5	27	12		419.0		349.5		402.0	
	11	502.5	43	13		418.0	0.300	341.5		390.5	0.238
	12	496.0	42	9	0.199	414.0		337.5			
	13	502.0	34	9		408.0				371.5	
	14	504.0	32	11			0.470			352.5	
	15	493.0	33	11		379.0	0.640	342.5		331.0	
	16		26	7		377.5	0.650	339.5	0.336	318.0	0.418
	17	464.5	26	7		375.0		320.5		316.5	0.413
	18	466.0	10	6	0.192	359.0	0.680	322.5	0.320	297.0	0.505
	19	452.5	11	4		340.5	0.880	307.0			
	20	434.5	7	17	0.350	321.5				265.0(dead)	0.485
	21	422.5	6	6		285.5(2 dead)	0.650	254.0	0.265		
	22	398.0	4	8	0.390	247.0(3 dead)	0.960	259.5	0.630		
	23		3	2				242.0			
	24	346.0	3	2	0.740			221.0	0.685		
	25	344.5	3	3				208.5 (dead)			
	26	327.5	2	1	0.700						
	27	309.0	3	1	0.945						
	28	288.5	1	3	0.785						
	29	275.5 (dead)	1	1	0.640						

Table 20.

Treatment	Body weight in g. beginning of experiment	Day of experiment on which fall in body wt. begins	Day of death or day of killing when moribund	Right adrenal weight in mg.	Right adrenal weight in mg. body wt. in g.
None: healthy	587			214.8	0.37
	542			156.0	0.23
	608			230.8	0.38
	655			188.2	0.29
	324			90.7	0.28
	328			76.2	0.23
Scorbutic diet: no treatment	507	12	21	202.8	0.34
	425	11	21		
	415	12	22		
	540	15	29	208.4	0.39
	500	13	29		
	435	12	21	121.4	0.25
	320	11	21	127.7	0.39
	305	9	25		
	300	9	25	182.9	0.52
	590	15	27	282.4	0.47
	545	15	27	326.4	0.50
	395	15	28	146.5	0.32
	320	15	28	184.8	0.47

Fig. 12. shows these results in group II.

1. All animals showed a fall in body weight beginning on the 9th to the 15th day after starting the scorbutic diet.

2. The daily food intake began to fall rapidly about the 12th to the 16th day after the scorbutic diet began.

3. Death from scurvy occurred in all animals on the 21st to the 29th day of the experiment. The typical features of acute scurvy have been adequately described by numerous workers and are only briefly summarised here. There was marked wasting. The animal frequently lay on its side in the typical "face-ache" position and tried to sit in feeding dishes as the wire mesh of the cage obviously hurt its feet. On examination the gums were

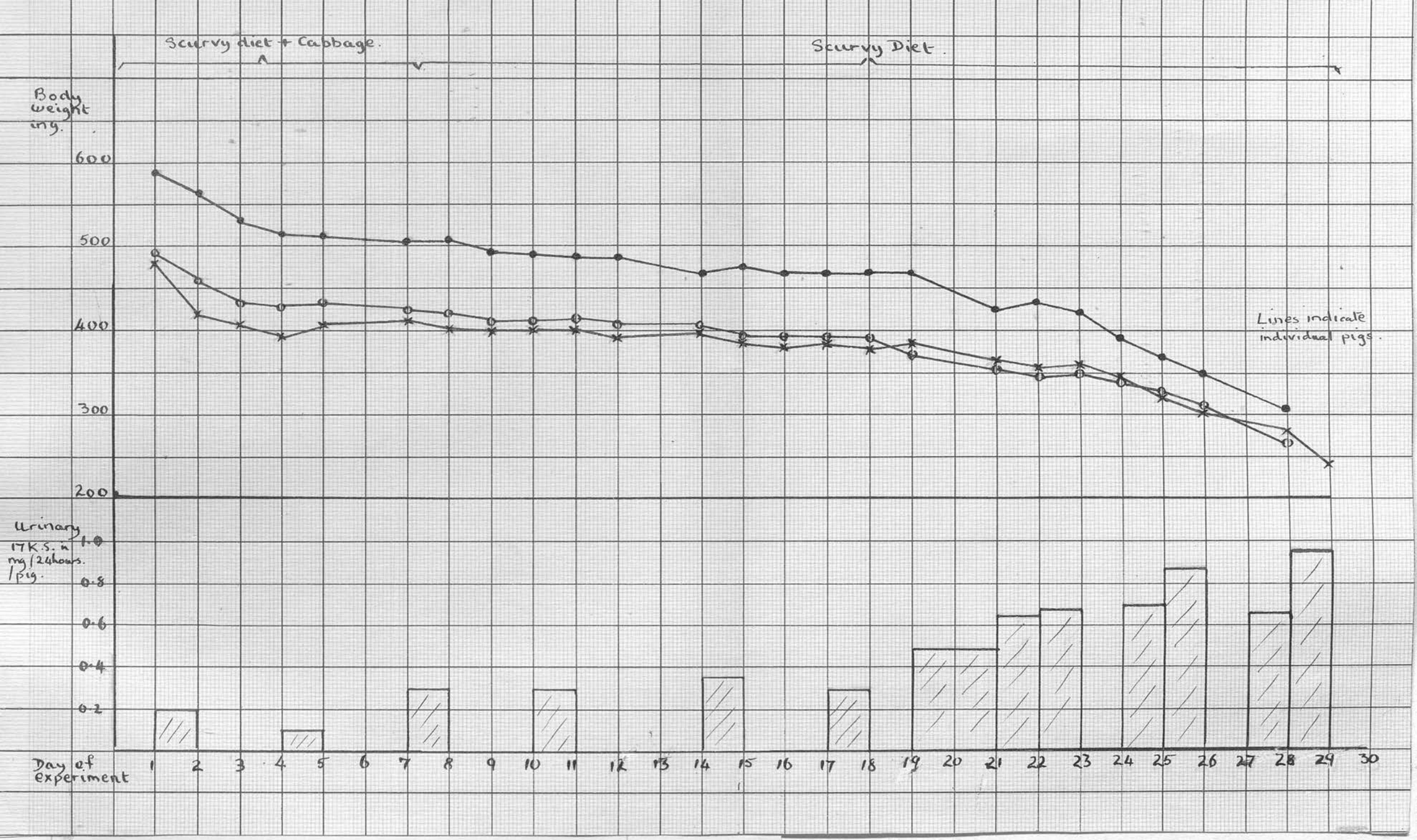


Figure 12. Body weights and urinary 17 - ketosteroid excretion in three guinea pigs receiving a scorbutic diet (Group II)

haemorrhagic and frequently there were black tarry stools together with dark red blood at the urethral orifice and vagina. Much haemorrhage was present in the gut, particularly in the large intestine and occasionally some red blood was present in the stomach. Much haemorrhage was often seen in the bladder too. The liver showed mild fatty degeneration. Haemorrhage in the adrenals was not often a feature in these animals. The kidneys rarely showed haemorrhage, but the demarkation between cortex and medulla was poor. Massive haemorrhages occurred terminally in the lungs and there was frequently a large pericardial effusion. Large subperiosteal haemorrhages occurred and at the epiphyseal lines the zone of ossification was broadened and dense, the regular arrangement of cells was lost, fragments of bone were scattered around and haemorrhages occurred.

4. In all groups of animals the urinary 17-ketosteroid excretion showed a gradual but definite increase of varying degree as the animals became scorbutic, and excretion reached a peak in the terminal phases. In general the excretion was greater in heavier (and therefore older) animals.

5. When not required for histological examination the right adrenal gland was weighed to the nearest 0.1 mg. Scorbutic glands showed an increase in weight over glands from healthy pigs, and the mean ratio of right adrenal gland (mg.) to highest body weight (g.) while on a scorbutic diet was 0.40 $\pm$ 0.03. Corresponding figures for healthy pigs are 0.30 $\pm$ 0.03 (see table 20).

6. The ascorbic acid content of the adrenal glands of healthy pigs varied between 106 and 164 mg/100 g. adrenal tissue. The ascorbic acid content of adrenal glands of grossly scorbutic animals was negligible and it could not be demonstrated histologically with a silver stain.

17-KETOSTEROID EXCRETION DURING CHRONIC SCURVY.

Methods.

Two male guinea pigs weighing 440 and 490 g. were placed on scurvy diet supplemented with cabbage. After four days the cabbage was stopped and thereafter they were given 2 mg. ascorbic acid in water by mouth every fourth day, from a pipette. Urinary 17-ketosteroids were determined at intervals.

Results.

1). The animals survived for sixty four and sixty seven days. During this time there was a gradual loss in weight (see fig. 13).

2). On examination the animals showed marked wasting, and swollen wrist joints. The pale liver showed fatty degeneration; there were no haemorrhages. The right adrenal gland to body weight ratio was 0.36.

Urinary 17-ketosteroids were as follows:-

Treatment	Day	Urinary 17-ketosteroids in mg/24 hrs/guinea pig.
Scurvy diet + cabbage		0.197
Scurvy diet + 2 mg. ascorbic acid every 4th day	11	0.170
	21	0.073
	28	0.143

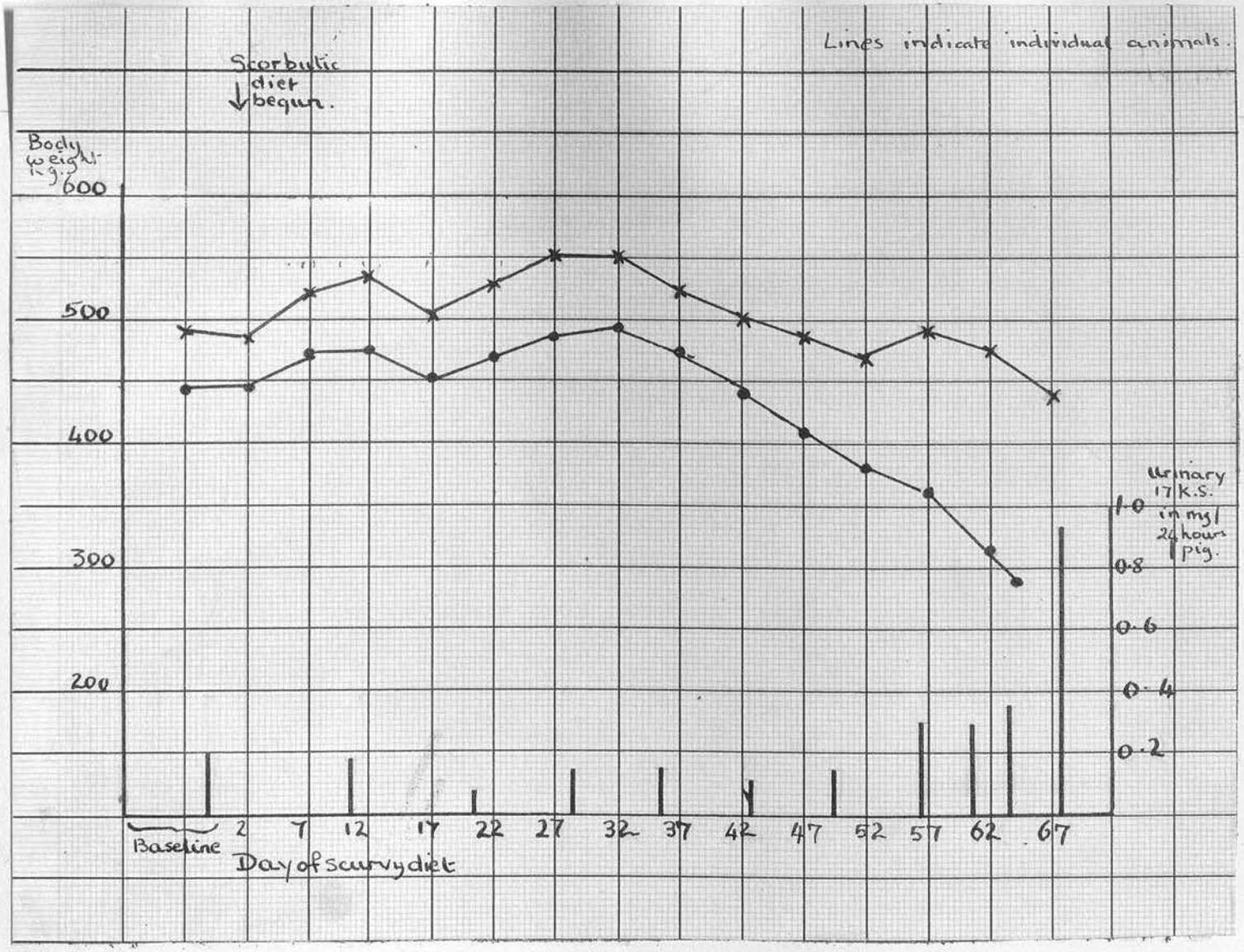


Figure 13. Body weights and urinary 17 - ketosteroid excretion in chronic scurvy.

Treatment	Day	Urinary 17-ketosteroids in mg/24 hrs/guinea pig.
	35	0.137
	42	0.100
	49	0.156
	56	0.291
	60	0.250
	63	0.353
	67	0.710

Thus excretion remained low until the 56th day, when it began to rise and showed a terminal increase.

INJECTION OF TURPENTINE AS AN EXAMPLE OF A STRESS IN THE GUINEA PIG.

The excretion of neutral 17-ketosteroids followihg a stress has been examined, to see whether it approached that found in the late stages of scurvy.

Method.

A pair of male guinea pigs (weighing 450 and 700 g.) were placed in a metabolism cage and after a preliminary base-line period, were injected with 0.5 ml. turpentine subcutaneously on the anterior abdominal wall.

Result.

The injection gave rise to an abscess which was at a maximum and well-localised, after eight days. It gradually resolved and the animals remained healthy.

Neutral 17-ketosteroid excretions(see fig. 14) in mg/24 hours/animal were as follows:-

Baseline period:		0.169
Days since turpentine injection:		
	Day 2	0.243
	3	0.223
	4	0.223

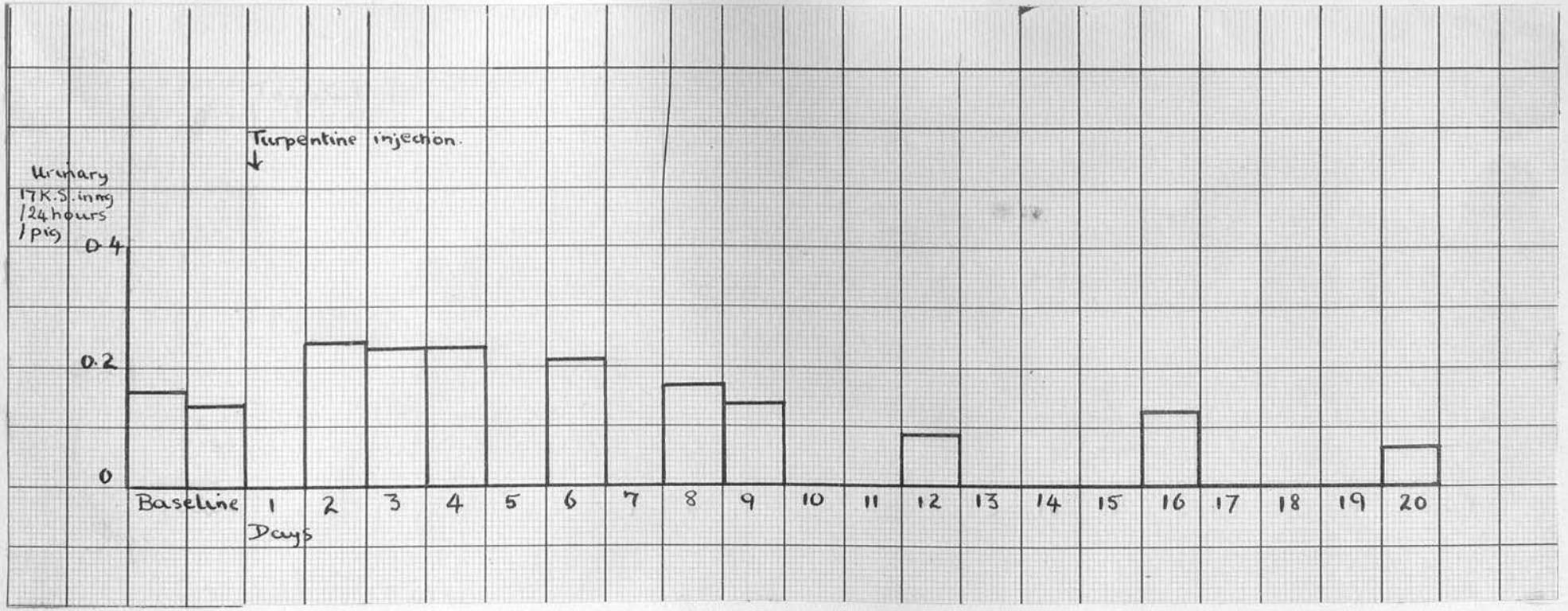


Figure 14. Urinary 17 - ketosteroid excretion following the stress of a subcutaneous injection of turpentine.

Day 6	0.214
8	0.178
9	0.145
12	0.091
16	0.132
20	0.074

After a preliminary rise, excretion has fallen and not returned to baseline levels for about sixteen days. The response has been small, and does not compare with that seen in the late stages of scurvy.

EFFECT OF ADRENALECTOMY ON THE DEVELOPMENT OF SCURVY.

Numerous workers have shown that the adrenal gland contains a very high concentration of ascorbic acid (Szent - Gyorgi 1928). Urinary 17-ketosteroids are thought to arise from the adrenal cortex, and to a lesser extent from the testes in the male. It therefore seemed of interest to investigate the development of scurvy in adrenalectomised animals.

Method.

Three separate experiments were carried out. In each case, animals were placed in metabolism cages grouped as follows:-

1). Two intact untreated animals.

2). Two intact animals receiving 2 mg. DOCA-in-oil daily.

3). One or two (see tables) adrenalectomised animals receiving 2 mg. DOCA daily. (The technique of adrenalectomy is described later).

The first experiment was on male animals, the second on females, and the third on males and only contained groups (2) and (3). The animals were maintained on a scorbutic diet

which was supplemented with cabbage for the first five days of the experiment. Urinary 17-ketosteroids were determined while the cabbage supplement was being given, and then at intervals after its withdrawal (see table 21), but always on alternate days in the last eight days while both animals were alive. In some instances, when a Sunday intervened, 48 hour collections were used. All animals were autopsied.

Results.

Details are shown in Table 21 .

1). Adrenalectomized guinea pigs deprived of ascorbic acid showed a rapid fall in body weight, and died after seven, three and five days. One survived for seventeen days, and at death a small round extra-adrenal gland (confirmed microscopically) weighing 29 mg. was found lying on the posterior abdominal wall in the angle between the right kidney and left adrenal vessels.

2). The urinary 17-ketosteroids in the adrenalectomized animals failed to show a rise before death unlike the intact guinea pigs.

3). Adrenalectomized animals had some mild haemorrhagic manifestations at death, though much less than in the intact animals. Small haemorrhages were present in the gums, body wall and gut, and occasionally to a slight extent in bladder and kidneys. Sections of the joints showed early epiphyseal changes consistent with a diagnosis of scurvy, but they were not nearly so extensive as those seen in the late stages of scurvy in the intact animal (fig. 15).

TABLE 21

DEVELOPMENT OF SCURVY IN ADRENALECTOMISED GUINEA PIGS.

URINARY 17-KETOSTEROID EXCRETION mg/24 hr. GUINEA PIG.

EXPERIMENT 1. Male.

Day	(1) Intact Untreated	(2) Intact Daily DOCA	(3) Adrenalectomised. Daily DOCA (1 guinea pig)
Prescurvy	Not done	0.27	0.18
Day of scurvy diet			
1	0.02	0.32	0.07
2		0.36	0.06
3	0.2	0.33	0.04
4		0.34	0.07
5	0.21	0.31	0.05
6, 7	0.18	0.48	0.03
12	0.21	0.58	
13,14		0.39	
16		0.93	
18		0.99	
20,21		0.66	
22	0.19		
24	0.14		
26	0.3		
27,28	Not done		
Day of death.			
Guinea pig A	36	27	7
Guinea pig B	29	21	
Ratio Rt. adrenal wt./mg. / Highest body weight in g.			
Guinea pig A.	0.43	0.53	
Guinea pig B.	0.50	0.46	

EXPERIMENT 2. Female.

Day	(1) Intact Untreated	(2) Intact Daily DOCA	(3) Adrenalectomised. Daily DOCA (2 guinea pigs)
	0.14	0.21	0.14
1		0.32	0.17
2	0.12	0.31	0.13
3	0.09	0.29	0.1 (one died)
9		0.36	
10			0.25
11		0.82	
12			0.26
13,14		0.63	0.22
15		1.03	
16			0.33
21	0.16		
23	0.38		
25	0.55		
27,28	0.53		
	30	25	17 (extra adrenal present)
	28	16	3
	0.38	0.60	
	0.41	0.50	

EXPERIMENT 3. Male.

Day	(1) Intact Daily DOCA	(2) Adrenalectomised Daily DOCA. (1 guinea pig)
	0.35	0.06
2	0.30	0.07
3		0.04
4,5		0.01
12	0.5	
17	0.38	
19	0.34	
20,21	0.38	
22,23	0.88	
	28	5
	23	

(a)

(b)

(c)

Figure 15.

(a) Normal epiphyses

(b) Epiphyses of an adrenalectomised guinea pig dying after seven days on a scorbutic diet.

(c) Epiphyses in acute scurvy.

x 100

4). Treatment of intact guinea pigs with DOCA during the development of scurvy did not depress the 17-ketosteroids. There was, in fact, a greater rise than usual in DOCA-treated animals and this was apparent throughout the development of scurvy. DOCA produced no change in haemorrhagic manifestations of the joints or the right adrenal weight to body weight ratio.

The autopsy findings on two adrenalectomized animals deprived of ascorbic acid were as follows:-

Male guinea pig (No. An) which died after 7 days.

Gums: Lower ones were very haemorrhagic.

Gut: No anal haemorrhage present. Two small haemorrhages were present on the greater curvature of the stomach, and very occasional small ones in the walls of the large and small intestines.

Kidneys: No haemorrhage.

Bladder: One small haemorrhage.

Liver: Smooth, slightly mottled, but no haemorrhages or fatty degeneration.

Spleen: Dark, very granular.

Thymus: Small residue present.

Lungs: Congested and haemorrhagic.

Female guinea pig (No. As) which died after three days deprivation of ascorbic acid.

Gums: Very small haemorrhage on lower gums.

Gut: No haemorrhage in stomach, or large intestine; one small haemorrhage in small intestine.

Kidneys: Pale; large left subcapsular haemorrhage; haemorrhage intra-perirenal fat on right side.

Bladder: No haemorrhage.

Liver: No fatty degeneration or haemorrhage.

Spleen: Dark, very granular.

Thymus: Small residue present.

Lungs: No haemorrhage.

CASTRATION AND ITS EFFECT ON 17-KETOSTEROIDS IN SCURVY.

The 17-ketosteroids are customarily considered to arise from the adrenal glands and testes in the male, and from the adrenals in the female, (Fraser, Forbes, Albright, Sulkowitch, & Reifenstein (1941)), though Huis in't Veld & Dingemanse (1952)have contributed evidence to suggest that the ovaries play a part too.

In view of the rise in steroid excretion during the later stages of acute scurvy, the position has been examined in castrated animals to see whether the gonads contribute significantly to the rise.

Method.

Five groups (two male and three female) of four animals each, were placed in metabolism cages; one pair of each group had been castrated fourteen to thirty five days previously. Urinary 17-ketosteroid determinations were performed on the second day after commencing a scorbutic diet, and on each alternate day during the last eight days when both animals of a pair were still alive.

Result.

The results are given in the following table:-

Table 22.

Guinea pig	Survival in days A	Survival in days B	Urinary 17-ketosteroids in mg/24 hours/animal. Day 2	Alternate days in the last 8 days when both animals were alive: 1	3	5	7
Gonadectomised. Male	28	29	0.08	0.34	0.47	0.30	1.0
Intact male.	30	25	0.19	0.26	0.20	0.23	0.62
Gonadectomised male.	28	24	0.12	0.22	0.21	0.24	0.62
Intact male.	28	28	0.12	0.15	0.14	0.27	0.28
Ovariectomised female.	24	24	0.14	0.40	0.28	0.44	0.67
Intact female	17	26	0.13	0.14	0.29	0.38	0.84
Ovariectomised female.	28	22	0.14	0.15	0.21	0.23	0.14
Intact female	27	29	0.17	0.17	0.41	0.32	0.66
Ovariectomised female	26	26	not due	0.32	0.26	0.74	0.67
Intact female	26	28		0.24	0.17	0.35	0.34

The excretion in castrated and intact animals is not significantly different. In view of experiments carried out on adrenalectomized animals, it would appear that the rise in excretion in scurvy is of adrenal origin, There is also no significant difference in the time of survival.

EFFECT OF ACTH IN SCURVY.

To discover whether ACTH effected survival time and the pathological manifestations in scurvy.

Method

Four male guinea pigs were placed in pairs in metabolism cages. ACTH administration to one pair was begun at the same

time as the scorbutic diet. ACTH was administered in physiological saline in a daily dose equivalent to 10 mg. La-1-A (Armour Batch No. H.7911) as four subcutaneous injections at two-hourly intervals. None was given on Sundays. Daily body weights, urine volumes and food intake were measured.

Results.

1. All animals showed a fall in body weight and ultimately died with manifestations of scurvy.

2. On the 27th day of the diet one of each pair was dead, and the second animal in each pair was moribund.

3. Food intake began to fall at the same time as the untreated scorbutic controls.

4. The pathological findings of scurvy were not modified by ACTH.

The ratios of right adrenal gland in mg. to highest body weight mg. were 0.44 and 0.47 in the treated and 0.47 and 0.50 in the untreated animals.

The influence of ACTH on survival time is summarised in Table 25 .

The 17-ketosteroid excretion in normal and scorbutic guinea pigs receiving daily ACTH.

Method.

ACTH administration was begun at the same time as the scorbutic diet to a pair of male guinea pigs. Each received the equivalent of 20 mg. La-1-A daily (Armour Batch No. H.7911)

as eight subcutaneous injections at hourly intervals. No ACTH was given on Sundays. Daily body weights, urine volume and food intake were measured and 17-ketosteroids were determined at frequent intervals. In addition, a second pair of animals received daily ACTH in the same dosage for the same length of time, while on a scurvy diet supplemented with cabbage.

Results.

These are summarised in figs. 16 and 17 .

1. The course of acute scurvy was unchanged and the guinea pigs died on the 18th and 22nd days. The steady fall in body weight was not checked, and there was no change in the pathological findings. The right adrenal gland to body weight ratios were 0.67 and 0.54. The pair receiving the cabbage supplement in addition, continued to gain weight and exhibited no unusual findings.

2. The urinary 17-ketosteroids were as follows:-

Scurvy diet + ACTH

Day	Excretion in mg./ 24 hours/animal.
1	0.362
4	0.124
6	0.890
8	0.940
12	1.955
14	1.04
18	0.90
19	0.930
20	1.07
21	1.77
22	1.06
24	0.485
25	1.55

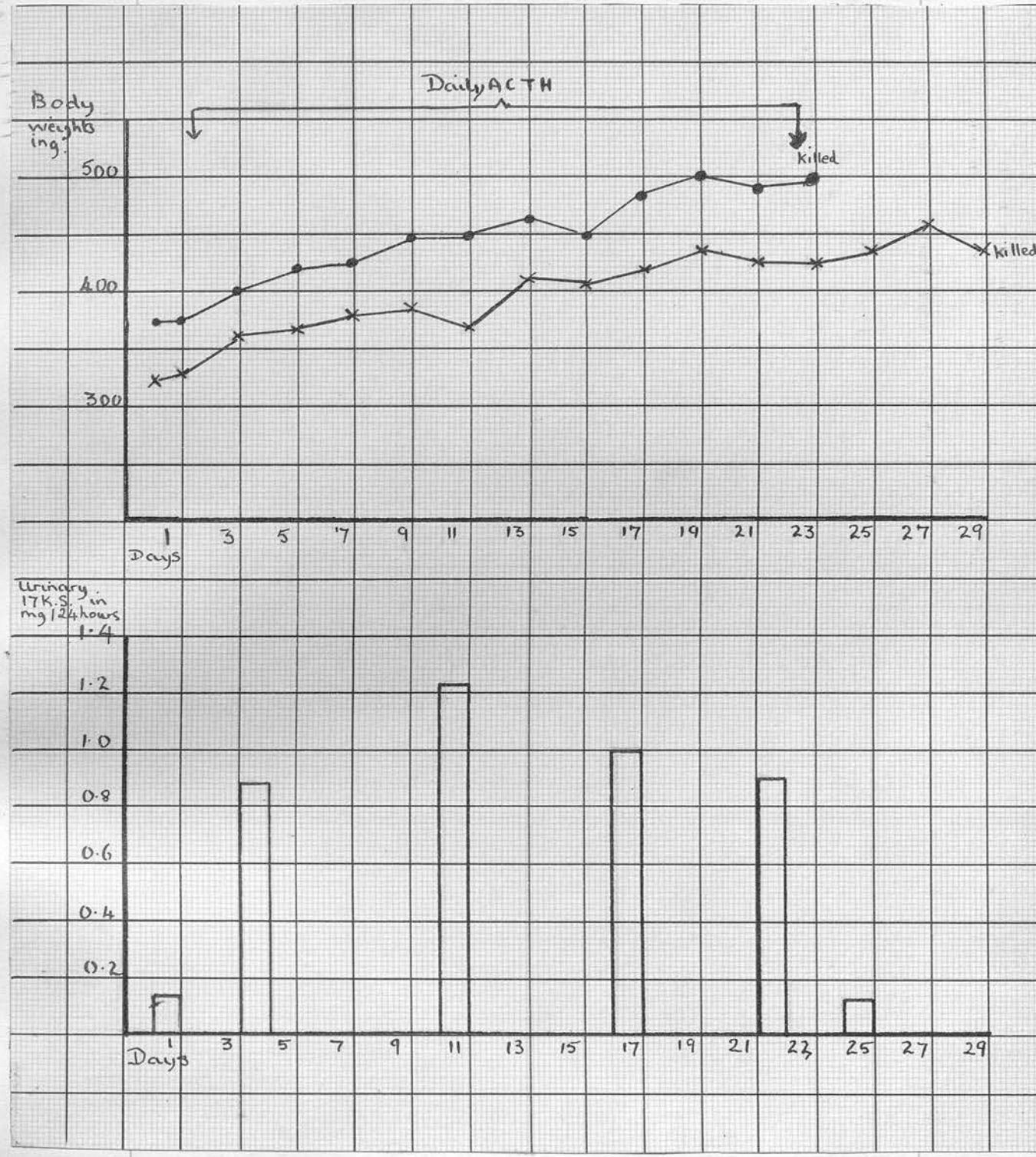


Figure 16. Body weights and urinary 17 - ketosteroid excretion in a pair of guinea pigs receiving daily ACTH; scurvy diet supplemented with cabbage.

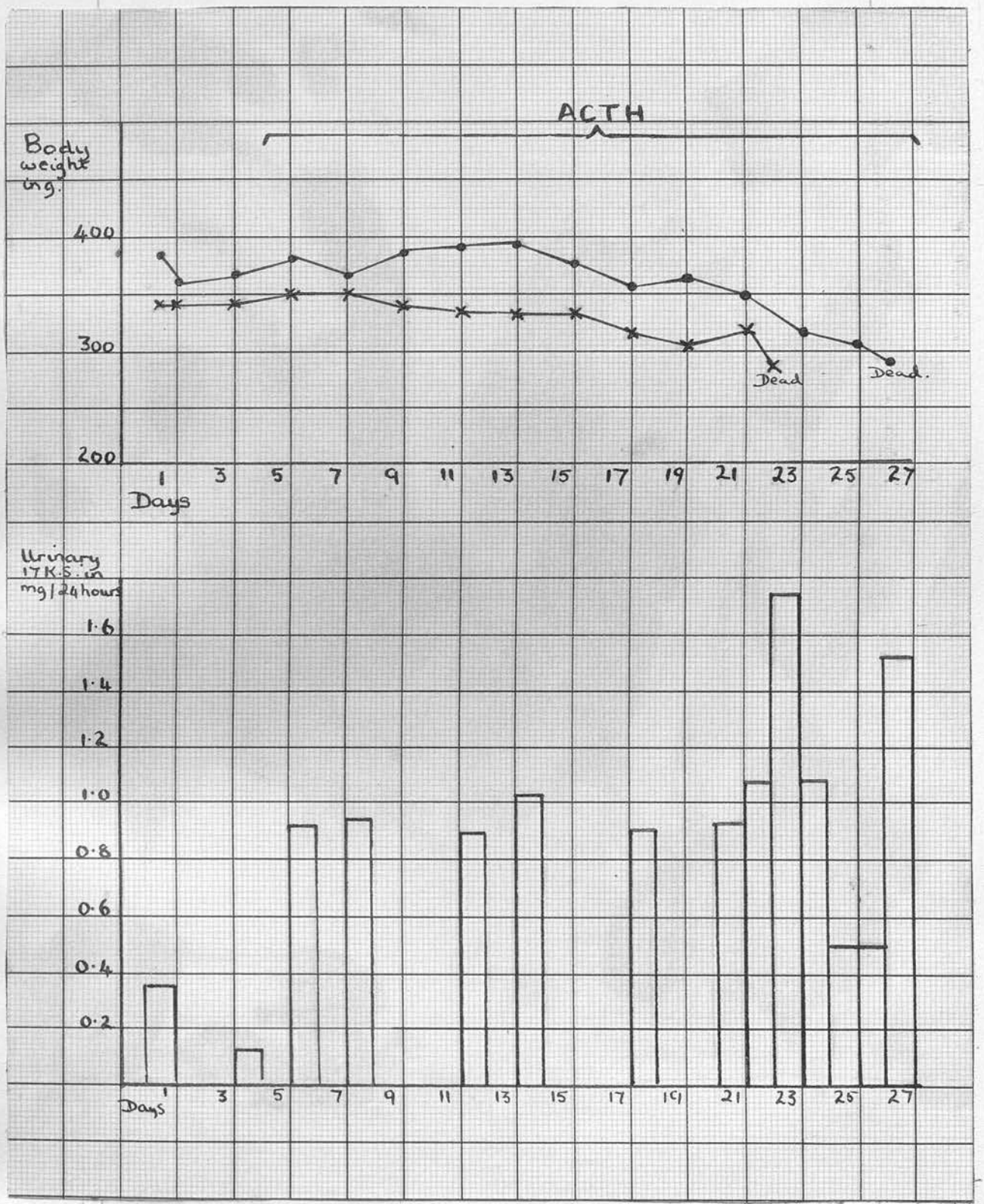


Figure 17. Daily ACTH administration to a pair of male guinea pigs on a scorbutic diet.

Scurvy diet + cabbage + ACTH

Day	Excretion in mg/ 24 hours/animal.
1	0.142
4	0.860
11	1.23
17	1.03
22	0.895
25	0.132

There was therefore no evidence that ACTH altered the course of acute scurvy. The 17-ketosteroid results, however, did suggest that the scorbutic animals (which had a lower negligible, adrenal ascorbic acid content) was still capable of responding to ACTH. During the first fifteen days or so of scorbutic diet a response to ACTH undoubtedly occurred. The high urine excretion of the grossly scorbutic animals could merely be the normal response to acute advancing scurvy. Experiments were then designed to settle this important point.

The Adrenal Ascorbic Acid at Various Stages of Scurvy.

Methods.

Six male and six female guinea pigs weighing 302 - 608 g. obtained from one registered breeder were maintained on the scorbutic diet. After twelve days on this diet, the adrenal glands of four animals were removed under ether anaesthesia, cleaned, dried with filter paper and weighed on a torsion balance. Each was ground with sand in 8.0 ml. 6% trichloracetic acid, and the ascorbic acid content was determined by the dinitrophenylhydrazine method of Roe and Kuether (1943). Similarly, both glands were removed from four animals after twenty-one days, and from three which were moribund in the

terminal stages of scurvy (one animal died overnight and is excluded). For comparison the adrenal ascorbic acid in three healthy male guinea pigs, receiving a supplement of cabbage was also determined.

Result.

The adrenal ascorbic acid concentrations in mg/100 g. tissue are given in Table 23. By the twenty-first day the amount of ascorbic acid was very low indeed and in the moribund animals negligible amounts were found.

Effect of Exogenous ACTH on Urinary 17-ketosteroid Excretion when the Adrenal Ascorbic Acid is Minimal.

Methods.

Six separate experiments were performed, two on female guinea pigs and four on males, varying in weight over a range of 341 to 643 g. Each experiment was as follows:-

Four guinea pigs in the same weight range were placed in pairs in two metabolism cages and maintained on a scorbutic diet. Body weights were determined daily. On the 19th to 22nd or 20th to 23rd day, inclusive of the scorbutic regime, one pair received ACTH, and the other pair received normal saline. The ACTH was administered in a dose equivalent to 20 mg.La-1-A in 4.0 mls. saline daily, as eight subcutaneous injections of 0.5 ml. at hourly intervals between 10 a.m. and 5 p.m. The saline was similarly administered as eight injections of 0.5 ml

Table 23.

ADRENAL ASCORBIC ACID DURING THE DEVELOPMENT OF SCURVY.

Description of guinea pigs.	Ascorbic acid right adrenal in mg/100g. tissue	Ascorbic acid left adrenal in mg/100g. tissue
HEALTHY		
(1) Male	106	106
(2) Male	154	139
(3) Male	164	118
AFTER 12 DAYS ON A SCORBUTIC DIET.		
(1) Female	13.3	10.6
(2) Female	9.6	15.7
(3) Male	nil	nil
(4) Male	22.9	19.5
AFTER 21 DAYS ON A SCORBUTIC DIET		
(1) Female	5.7	3.6
(2) Female	0.5	0.4
(3) Male	0.5	nil
(4) Male	nil	nil

ACUTE SCURVY, MORIBUND.	Ascorbic acid right & left adrenal in mg/100 g. tissue.
Female	0.1
Female	nil
Male	0.1

at hourly intervals. The previous experiment had shown that the adrenal ascorbic acid was very low at this stage. Twenty-four hour specimens of urine were collected over HCl on the day before injection, during the period of injection and for the twenty-four hours (or forty-eight hours if a Sunday intervened) following injection. Urinary 17-ketosteroids in each specimen were determined as already described. The animals were then left to develop scurvy, and when moribund were killed with ether and autopsied. The right adrenal gland was removed and weighed and particular note was taken of the extent of any haemorrhage.

Result.

Table 24 shows the urinary 17-ketosteroid excretion on each day. It also shows the total amount of 17-ketosteroids in mg. produced during the period of injection and the percentage increase in 17-ketosteroids with ACTH over the four days of treatment as compared with the excretion during injection with saline. This table also gives the time of survival of each animal and the ratio of the weight of the right adrenal gland in mg. to the highest body weight recorded during the experiment. Fig. 18 summarises the results of steroid excretion.

1). In all experiments, ACTH led to an increase in 17-ketosteroid excretion, and the increase with ACTH as compared with saline varied from 40 to 206%. The highest excretion recorded was 1.1 mg/24 hours. There was a tendency for a slight rise to occur during the period when saline only was

TABLE 24

Experiment	NEUTRAL 17-KETOSTEROIDS IN MG/24hr/ANIMAL									
		During injection								
	Day before ACTH or saline treatment	Day 1	Day 2	Day 3	Day 4	Day following ACTH or saline treatment	17 ketosteroids in mg. produced during the injection period.	% Increase in 17-ketosteroids with ACTH	Ratio right adrenal weight in mg. Highest body weight in g.	Total survival in days	Survival after the period of injection (in days)
1. Female										
Saline	0.15	0.21	0.22	0.41	0.30	0.19	1.14	174	0.48	31	9
									0.39	31	9
ACTH	0.27	0.70	0.97	0.86	0.60	0.51	3.13		0.49	31	9
									0.83	31	9
2. Female										
Saline	0.18	0.30	0.40	0.34	0.47	0.52	1.51	113	0.65	32	9
									0.62	28	5
ACTH	0.12	1.00	0.66 (1 died)	0.46	1.10	0.42	3.22		0.59	22	nil
									0.71	29	6
3. Female										
Saline	0.21	0.34	0.51	0.53	0.30 (1 died)	0.08	1.68	45	0.34	29	6
									0.37	23	nil
ACTH	0.13	0.43	0.48	0.68	0.86	0.45	2.45		0.31	24	1
									0.43	26	3
4. Male										
Saline	0.09	0.19	0.50	0.21	0.25	0.24	1.15	206	0.37	29	7
									0.52	32	10
ACTH	0.23	1.02	1.03	0.71	0.76	0.38	3.52		0.39	33	11
									0.47	34	12
5. Male										
Saline	0.20	0.35	0.37	0.28	0.44	0.42	1.44	40	0.55	27	5
									0.43	25	3
ACTH	0.15	0.45	0.64	0.47	0.46	0.27	2.02		0.33	39	7
									0.59	28	6
6. Male										
Saline	0.20	0.34	0.35	0.19	0.41	0.58	1.29	48	0.44	25	3
									0.40	25	3
ACTH	0.12	0.32	0.53	0.47	0.59	0.28	1.9		0.46	31	9
									0.47	29	7

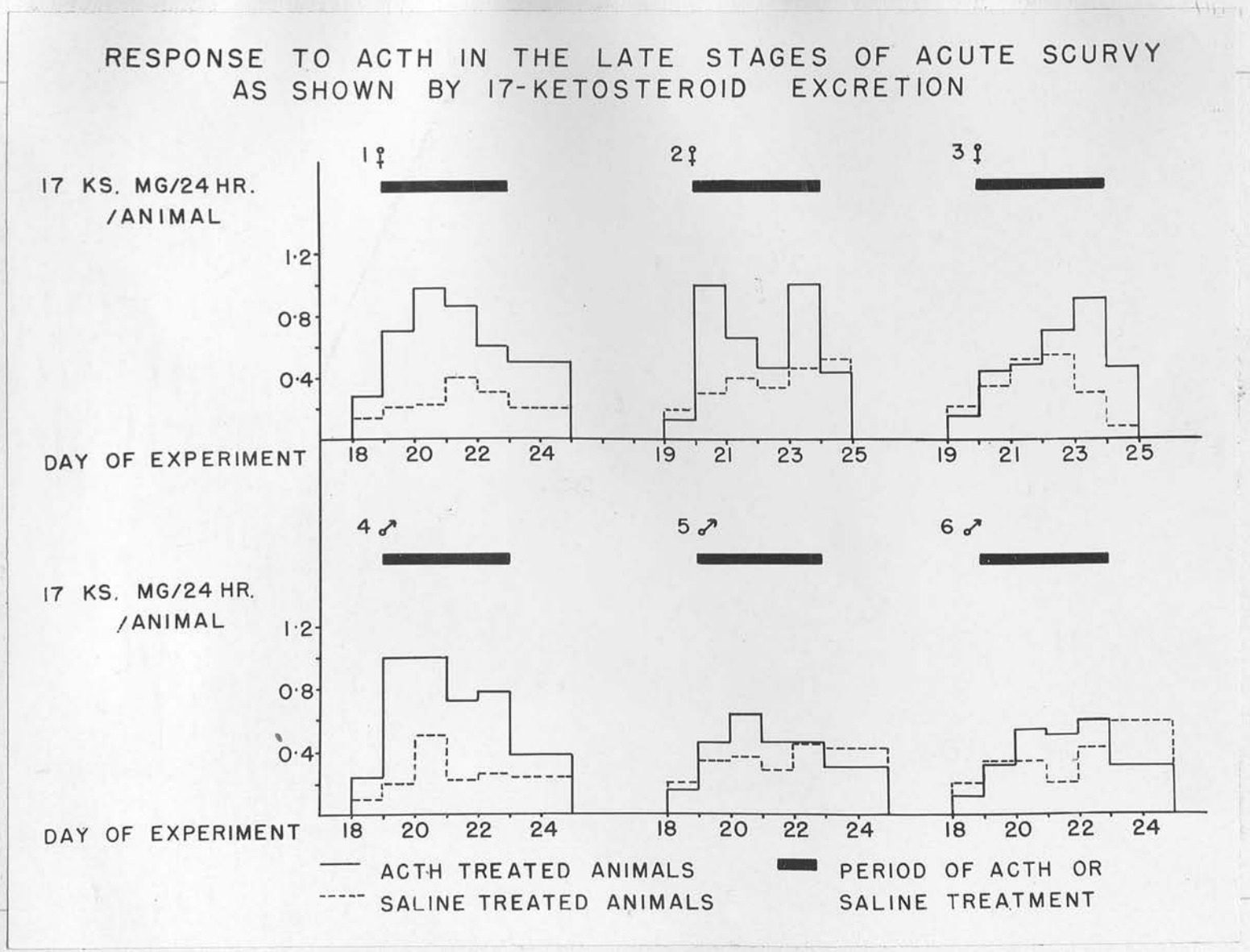

RESPONSE TO ACTH IN THE LATE STAGES OF ACUTE SCURVY
AS SHOWN BY 17-KETOSTEROID EXCRETION
17 KS. MG/24 HR. /ANIMAL
1♀
2♀
3♀
1·2
0·8
0·4
DAY OF EXPERIMENT
18 20 22 24
19 21 23 25
19 21 23 25
4♂
5♂
6♂
17 KS. MG/24 HR. /ANIMAL
1·2
0·8
0·4
DAY OF EXPERIMENT
18 20 22 24
18 20 22 24
18 20 22 24
ACTH TREATED ANIMALS
SALINE TREATED ANIMALS
PERIOD OF ACTH OR SALINE TREATMENT

Figure 18.

injected, but this was probably not significantly different from the behaviour of uninjected scorbutic animals.

2). All animals were showing a fall in body weight when injections were begun, and this was unchanged by treatment.

3). The mean survival was 28.1 ± 0.8 days and 28.0 ± 1.1 days in those treated with saline and ACTH respectively, so this short period of treatment had no effect on ultimate survival.

4). In general, haemorrhagic manifestations though difficult to assess were somewhat more severe in those treated with ACTH. Haemorrhages were often more severe in the gut and bladder, more frequently seen in the adrenal glands than usual, and also occasionally present in the kidneys, pancreas and liver - rare sites for haemorrhage in the saline-treated animals.

5). Evidence of adrenal cortical hypertrophy in response to ACTH over and above that due to scurvy was not consistently obtained since the hormone was not administered for several days before the animals died and the glands could be examined.

EFFECT OF CORTISONE IN SCURVY.

Effect of 5mg. Cortisone daily on Normal and Scorbutic Guinea Pigs being fed ad lib.

Method.

A pair of male and a pair of female guinea pigs weighing about 400 g. were placed on scurvy diet ad lib and given one injection of cortisone daily in a dosage of 5 mg. (Merck's

suspension) subcutaneously (except on Sundays). Daily body weights were determined together with urine volumes, food intake and periodic 17-ketosteroid determinations. Cortisone was also administered to a pair of female animals maintained on scurvy diet supplemented with cabbage.

Result.

1). In animals on the scurvy diet only, the fall in body weight was unchanged by cortisone, and the subsequent pathological manifestations were the same as those of untreated scorbutic animals except for an increase in fatty degeneration of the liver. Haemorrhage was not diminished in these animals.

2). The administration of cortisone prevented the increase in weight of the adrenal glands normally found in scurvy. (see table 25). No ascorbic acid could be demonstrated histologically in their scorbutic glands.

3). Urinary 17-ketosteroid excretion was as follows:-

	Treatment	Day	17-ketosteroid excretion in mg/ 24 hour/animal.
I	Scurvy diet	2	0.210
	+ cabbage	6	0.320
	Females	13	0.365
		21	0.335
		22	0.385
		27	0.453
II	Scurvy diet	2	0.090
	Females.	5	0.125
		8	0.150
		11	0.130
		14	0.113
		17	0.232
		19	0.219
		21	0.119

	Treatment	Day	17-ketosteroid excretion in mg/ 24 hour/animal
III	Scurvy diet Males.	4	0.189
		7	0.216
		10	0.175
		13	0.121
		17	0.200
		20	0.248
		22	0.092
		24	0.261

These results suggest that cortisone in a daily dosage of 5 mg. suppressed the endogenous urinary 17-ketosteroid excretion and no large terminal rise has occurred in the scorbutic animals.

Effect of 20 mg. Cortisone Daily on Normal and Scorbutic Guinea Pigs fed ad lib.

Method.

Four male guinea pigs were placed in pairs in metabolism cages. Both pairs were placed on a scorbutic diet ad lib, and in one pair it was supplemented with cabbage. All animals received 20 mg. cortisone once daily subcutaneously (except on Sundays). Records of body weight, food intake and urine volume were kept.

Result.

1). In the animals on the scurvy diet, the fall in body weight was unchanged, and they died with typical manifestations of scurvy; haemorrhage was not diminished noticeably, but fatty degeneration of the liver was increased.

2). Administration of cortisone prevented the increase in weight of the adrenal glands normally found in scurvy (see

table) and also reduced the adrenal size in the healthy animals. No ascorbic acid could be demonstrated histologically in the treated scorbutic adrenal glands.

3). Urinary 17-ketosteroid excretion was as follows:-

	Treatment	Day	17-ketosteroid excretion in mg/ 24 hour/animal.
I	Scurvy diet Males.	2	0.378
		5	0.645
		9	1.58
		14	1.08
		18	0.62
		19	1.21
		20	0.460
		21	0.61
		22	0.625
I	Scurvy diet + cabbage Males.	1	0.15
		6	0.29
		13	1.67
		21	1.60
		22	1.20
		25	0.575
		29	0.580

Thus a daily dosage of 20 mg. raised the excretion to about 1 mg. in control and scorbutic pigs. Sprague et al (1950) suggested that 17-ketosteroids which appear in the urine during cortisone administration are largely derived from administered cortisone.

Effect of Cortisone in Scurvy, when Paired - Feeding is Employed.

Since cortisone is known to increase appetite, an experiment was carried out in which cortisone was administered to animals paired - fed with untreated scorbutic guinea pigs.

Method.

Four female guinea pigs (Group Bs) weighing about 500 g. were placed on a scorbutic diet, and 5 mg. daily cortisone subcutaneously was administered to two of them. The daily food intake of the untreated pair (Group I) was measured, and the cortisone - treated animals (Group 2) were paired - fed with them. Body weights were determined daily, and periodic 17-ketosteroid determinations were carried out.

Result.

1). The animals all lost weight at the same rate, and died with the manifestations of acute scurvy after 26 to 29 days.

2). The urinary 17-ketosteroids were as follows:-

(Group 1)
In the untreated scorbutic animals
(in mg/24 hours/animal)

Day 3	0.176
6	0.119
8	0.204
12	0.119
18	0.192
20	0.350
22	0.390
24	0.740
26	0.700
27	0.945
28	0.785
29	0.64

(Group 2)
In the cortisone - treated scorbutic animals

Day 8	0.181
11	0.102
14	0.340
17	0.370
19	0.240
22	0.440
24	0.390
26	0.456
28	0.655

Thus, depression of the urinary 17-ketosteroids by cortisone was not particularly marked in these paired - fed animals in contrast to the depression seen in the previous experiments with cortisone.

This experiment was therefore repeated using three groups of animals:-

1). Scurvy diet only.

2). Scurvy diet ad lib. + 5 mg. cortisone daily.

3). Scurvy diet, paired - fed with (1) and 5 mg. cortisone daily.

The 17-ketosteroid results were as follows:-

Group 1

Day 2	0.103
13	0.139
19	0.350
21	0.180
23	0.470
26	0.109
28	0.650
29	0.700

Group 2

Day 13	0.380
17	0.166
19	0.073
21	0.116
23	0.390

Group 3.

Day 2	0.33
13	0.41
15	0.264
17, 18	0.206
19	0.179
21	0.255
27	0.38
29	0.37

The adrenal weight to body weight ratios were:-

Group (1) 0.60 : 0.56

Group (2) 0.42 : 0.47

Group (3) 0.40 : 0.37

Group (1) shows the typical rise in 17-ketosteroids of acute scurvy. Both groups (2) and (3) show a depression in comparison, and this appears to be a little less marked in Group (3), which was paired - fed, as compared with Group (2) which was fed ad lib.

A number of similar experiments would have to be performed to obtain a sufficient number of figures to be certain that depression is less marked in paired - fed animals, but the two experiments already carried out are suggestive.

The technique of paired - feeding.

Scurvy diet and water are supplied ad lib to one group in troughs and the intakes are measured. On any particular day the paired group is given exactly the same amount of good and water as the ad lib group ate and drank on the previous day.

An example from Group Bs just described is given:-

June 16 - 17

Group (1)

1) Weight of dish + food = 900 g.

2) Weight of dish + food after 24 hours = 817 g.

3) Weight of food eaten = 83 g.

a) Volume of water given = 250 ml.

b) Volume of water left = 148 ml.

c) Volume of water drunk = 102 ml.

June 17 - 18

Group (1)

1)	900 g.	a) 250 ml.
2)	878 g.	b) 160 ml.
3)	22 g.	c) 90 ml.

Group (2)

1)	860 g.	a) 102 ml.
2)	777 g.	b) 0
3)	83 g.	c) 102 ml.

June 18 - 19

Group (1)

1)	850 g.	a) 200 ml.
2)	762 g.	b) 102 ml.
3)	88 g.	c) 98 ml.

Group (2)

1)	799 g.	a) 90 ml.
2)	777 g.	b) 0
3)	22 g.	c) 90 ml.

Summary of the Effect of ACTH and Cortisone on Survival.

The influence of cortisone and ACTH on the time at which the body weight begins to fall, survival time and adrenal weight is shown in Table 25 .

Table 25.

Treatment	Body weight in g. beginning of experiment	Day of experiment on which fall in body wt. begins	Day of death or day of killing when moribund	Right adrenal weight in mg.	Right adrenal weight weight in mg. body wt. in g.*
None:	587			214.8	0.37
healthy	542			156.0	0.23
	608			230.8	0.38
	655			188.2	0.29
	324			90.7	0.28
	328			76.2	0.23
Scorbutic	507	12	21	202.8	0.34
diet: no	425	11	21		
treatment	415	12	22		
	540	15	29	208.4	0.39
	500	13	29		
	435	12	21	121.4	0.25
	320	11	21	127.7	0.39
	305	9	25		
	300	9	25	182.9	0.52
	590	15	27	282.4	0.47
	545	15	27	326.4	0.50
	395	15	28	146.5	0.32
	320	15	28	184.8	0.47
Scorbutic	525	17	28		
diet: 5 mg.	470	17	28		
cortisone	345	17	24	126.1	0.36
daily	445	17	24		
	415	14	22	118.5	0.29
	380	14	21		
Scorbutic	620	12	22	172.2	0.28
diet 20 mg.	485	12	21	139.7	0.29
cortisone daily					
Scorbutic	385	10	22	259.0	0.67
diet 20 mg.	340	11	18	186.6	0.54
ACTH daily					
Scorbutic	710	15	27	311.5	0.44
diet; 10 mg.	585	17	27	285.5	0.47
ACTH daily					

* Body weight is the maximum recorded during the experiment prior to ACTH or cortisone treatment.

Applying a "t" test for the day on which the fall in body weight began, the 95% confidence limits for the mean difference are - 5.012 or + 1.578 - that is, it is unlikely that treatment increased the time of the first loss of weight by more than five days, or decreased it by more than one and a half days. The 95% confidence limits for the mean difference in survival time are - 3.019 and + 5.019 so that similarly it was unlikely that treatment increased the survival time by more than three days, or decreased it by more than five days. No definite antiscorbutic effect has therefore been demonstrated.

17-KETOSTEROID CONTENT OF FAECES AND URINE.

It was reported by Gallagher, Fukushima, Barry and Dobriner (1951) that rat faeces contained a large amount of neutral 17-ketosteroids. It was decided that a short experiment should be performed on a rat and a guinea pig to see whether a large daily faecal excretion was being missed in our experiments as only urines were being examined.

Method.

A male Wistar rat (200 g,) and a male guinea pig (400 g.) were placed in metabolism cages. Urine was collected in the usual way. Faeces were collected on cotton gauze, and after weighing were placed in 20.0 ml. 10% hydrochloric acid and homogenized before hydrolysing and extracting as for urine.

The animals were removed from their cages for half an hour twice a day for feeding, since contamination of faeces with food led to a high fat content in the final neutral 17-ketosteroid extract. Feeding was carried out in cages with glass bottoms so that any urine passed could be collected with a pipette.

Neutral 17-ketosteroids were determined before treatment, then after ACTH by injection, and cortisone by mouth and by injection. Dosage was as follows:-

1). ACTH. Guinea pig - 30 mg. La-1-A (Armour batch H.7911) daily for two days, as eight subcutaneous saline injections daily, at hourly intervals.

Rat - 15 mg. La-1-A (Armour Batch H.7911) daily for two days, as three intraperitoneal injections per day. The ACTH was administered in saline containing 0.25% glacial acetic acid.

2). Cortisone per os - 100 mg. in tablet form to each animal. The tablets were mixed with a little dried milk and water.

3). Cortisone by injection - 50 mg. subcutaneously to each animal.

Excretion was allowed to return to baseline levels between treatments.

Result.

Faecal and urine excretion in mg/24 hr hours/animal was as follows:-

	Rat		Guinea pig	
	Faeces	Urine	Faeces	Urine
Baseline	0.032	0.033	0.037	0.350
After 2 days ACTH	0.075	0.009	0.123	0.424
After cortisone per os. Day 1.	0.110	0.085	specimens lost	
Day 2	0.240	0.080	0.071	0.620
Day 3	0.115	0.028	0.028	0.410
After cortisone by injection.				
Day 1	0.064	0.057	0.130	2.30
Day 2	0.115	0.66	0.034	0.73

These results indicate that the amount of material in the faeces reacting as a 17-ketosteroid is very small, so that it appears unlikely that any large excretion has been missed in experiments performed.

Absorption curves of faecal and urine extracts.

The Zimmermann colour reaction for ketosteroids was carried out on the neutral lipids of guinea pig and rat faeces both before and after treatment with the Girard reagent. Extracts from the baseline period, after ACTH and after cortisone per os and by injection were examined. The absorption spectra of the colour produced by representative extracts were measured between 400 and 620 mμ. (fig. 19). Guinea pig urine extracts (curve B) gave absorption curves very similar to those obtained with a pure 17-ketosteroid - e.g. androsterone (curve A) with a maximum at 520 mμ. This does not of course prove

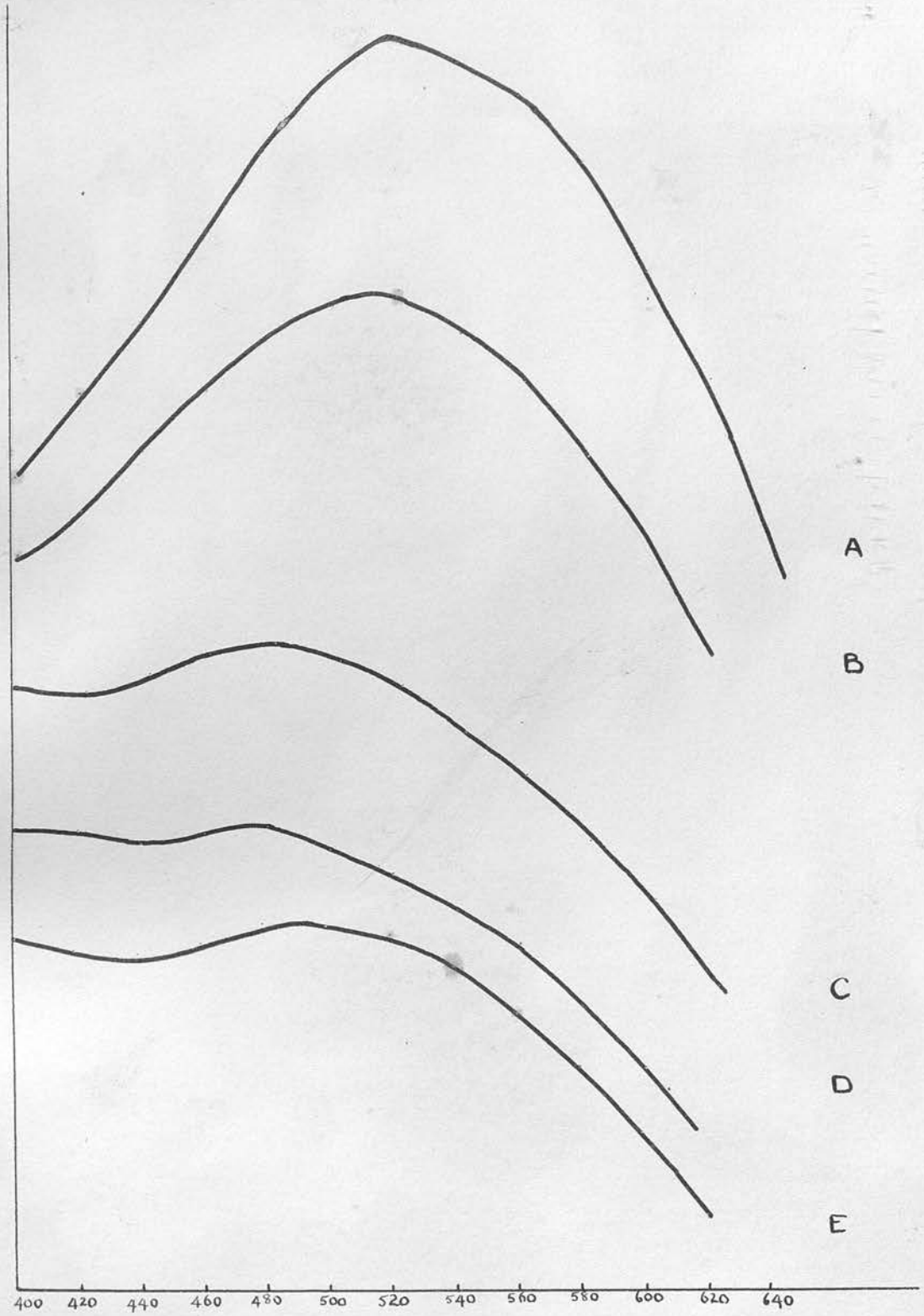


Figure 19 . Absorption curves.

a). Androsterone

b). Ketonic fraction of urine from a guinea pig treated with ACTH.

c). Ketonic fraction of urine from a rat treated with ACTH.

d). Ketonic fraction of faeces from a guinea pig treated with ACTH.

e). Ketonic fraction of faeces from a rat treated with cortisone

that the material present was 17-ketosteroid but is a strong indication.

Extracts of rat urine and of faeces (both guinea pig and rat) gave absorption spectra of a different type (curves C, D and E). Whilst there was a suggestion of a maximum at 500-520 mμ. there was considerable absorption over the whole range from 400-500 mμ. (i.e. the curves were almost flat). The nature of the material absorbing in this region is unknown.

THE EFFECT OF CORTISONE ON BODY WEIGHT IN CHRONIC SCURVY.

In 1950, Schaffenburg, Masson & Corcoran reported that guinea pigs on a scorbutic diet and receiving daily cortisone, gained weight at the same rate as untreated scorbutic controls. Unfortunately they terminated their experiment at twenty-one days, so ultimate survival figures are not available. The effect on body weight, however, was so striking that it was decided to repeat their experiment with some modifications, and also to carry it to its ultimate conclusion. Their scorbutic diet was supplemented by small doses of orange juice per os so that the animals ultimately developed chronic scurvy. In our experiment, it was decided that pure ascorbic acid should be used instead of orange juice.

Method.

Three groups of guinea pigs each containing four animals were placed on a scorbutic diet and treated as follows:-

Group (1) 5 mg. ascorbic acid daily by mouth in distilled water from a pipette.

Group (2) 1 mg. ascorbic acid every fourth day by mouth in distilled water from a pipette.

Group (3) Ascorbic acid as in Group (2) and cortisone subcutaneously once in a dosage of 5 mg/day, increased to 7.5 mg. on the 12th day and to 10 mg. on the 19th day of the experiment.

Body weights were measured daily, and all animals were examined at death, and the right adrenal gland was weighed. The ascorbic acid content of the right adrenal gland was determined by the method of Roe and Kuether (1943) in group (1).

Result.

The body weights are given in fig. 20.

1). Group (1) continued to gain in weight and were ultimately killed after forty six days.

2). Group (2) gained weight at first but ultimately died or were killed because they were moribund after twenty six to forty six days. The mean day at which the fall in body weight began was nineteen. At autopsy all animals showed haemorrhagic manifestations.

3). Group (3) gained in weight but ultimately died after thirty three to forty three days. The mean day at which the fall in body weight began was twenty. At autopsy the general impression was that haemorrhagic manifestations were much reduced in these animals; the increase in the size of the adrenals was prevented by cortisone.

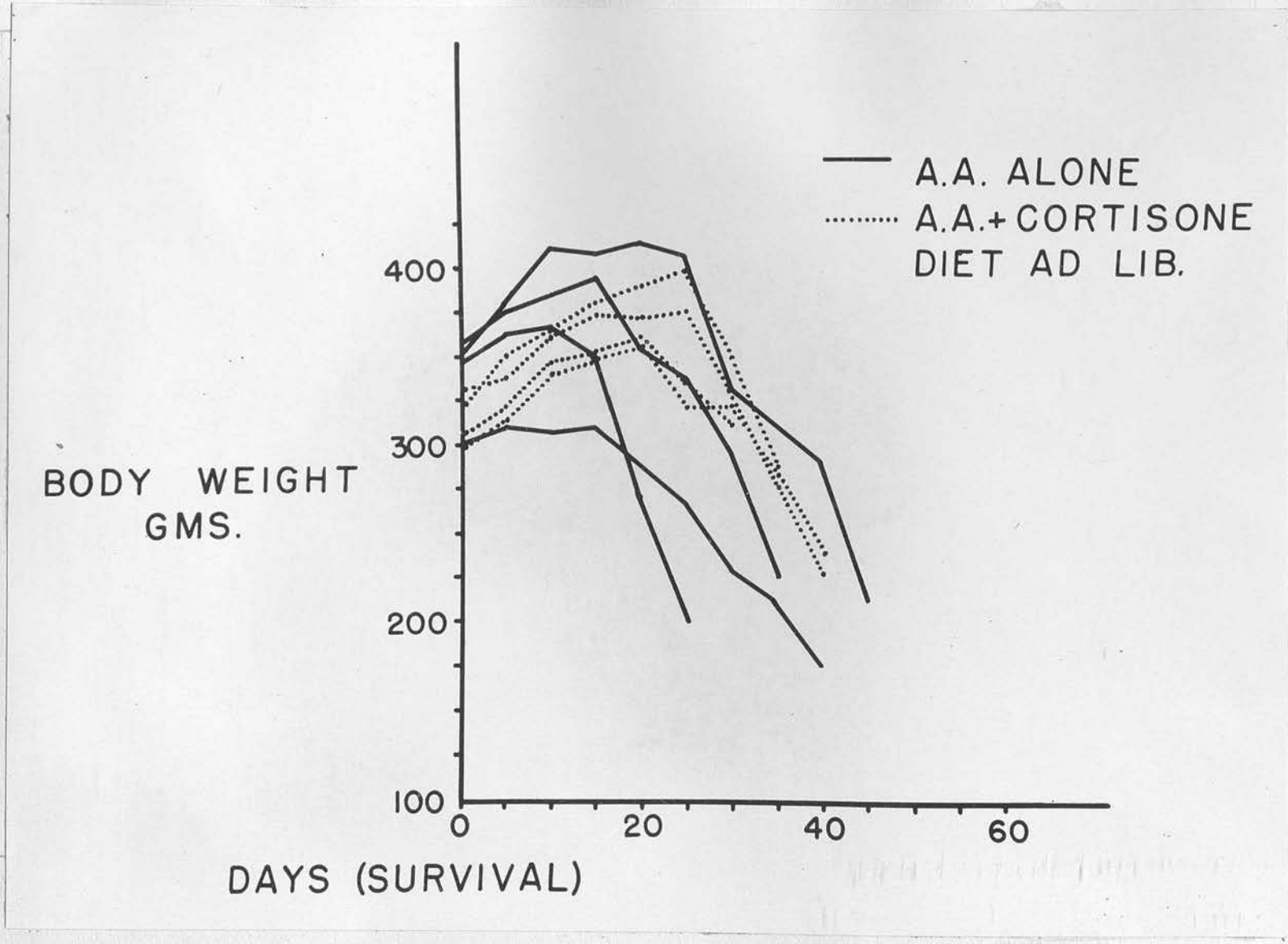


Figure 20. Growth curves of guinea pigs maintained on a scorbutic diet, supplemented with 1 mg. ascorbic acid per os every 4th day. One group received cortisone. All animals were fed ad lib.

4). Further details are given in the following table:-

Table 26.

Guinea pig	Day of death	Ratio Rt. adrenal wt. in mg. / Highest body wt. in g.	Rt. adrenal ascorbic acid in mg/100 g.
Group (1)			
A Male	Killed after 46 days	0.29	30.8
B Male	"	0.28	31.0
C Female	"	0.35	42.7
D Female	"	0.35	46.0
Group (2)			
A Male	46	0.57	
B Male	40	0.48	
C Female	26	0.53	
D Female	35	0.50	
Group (3)			
A Female	33	0.25	
B Male	42	0.32	
C Female	43	0.30	
D Female	38	0.50	

Thus cortisone has failed to influence ultimate body weight and survival. It has reduced haemorrhagic manifestations and adrenal size. The finding of Schaffenberg et al (1950) has, therefore, not been confirmed.

In view of the fact that cortisone is known to influence appetite, the previous experiment was performed again with modifications.

Method.

Two groups of four male animals each, were set up as follows:-

Group (1) received scurvy diet and water ad lib but the daily intake was measured. They also received 1 mg. ascorbic acid per os every fourth day.

Group (2) were paired - fed with group (1). They received 1 mg. ascorbic acid per os every fourth day, and one daily subcutaneous injection of cortisone in a dosage of 5 mg. increased to 7.5 mg. on the twelth day and to 10.0 mg. on the nineteenth day of the experiment.

Result.

1). The changes in body weight are given in fig. 21. Three out of four cortisone treated animals showed a minimal rise in the first five days only. Thereafter they showed a steady fall in weight, and the difference in the weight curves of the two groups is very striking. Survival in the two groups was virtually the same.

2). Haemorrhagic manifestations were mild in both groups, but very much less in the cortisone treated animals. Cortisone depressed the adrenal gland to body weight ratios.

3). Survival and adrenal gland to body weight ratios were:-

Table 27.

Guinea pig	Day of death	Ratio Right adrenal gland mg. / Highest body weight in g.
1 mg. ascorbic acid every 4th day		
A.Male	35	0.52
B.Male	32	0.47
C.Male	55	0.38
D.Male	36	0.43
1 mg. ascorbic acid every 4th day + cortisone.		
A.Male	42	0.24
B.Male	31	0.30
C.Male	37	0.18
D.Male	31	0.23

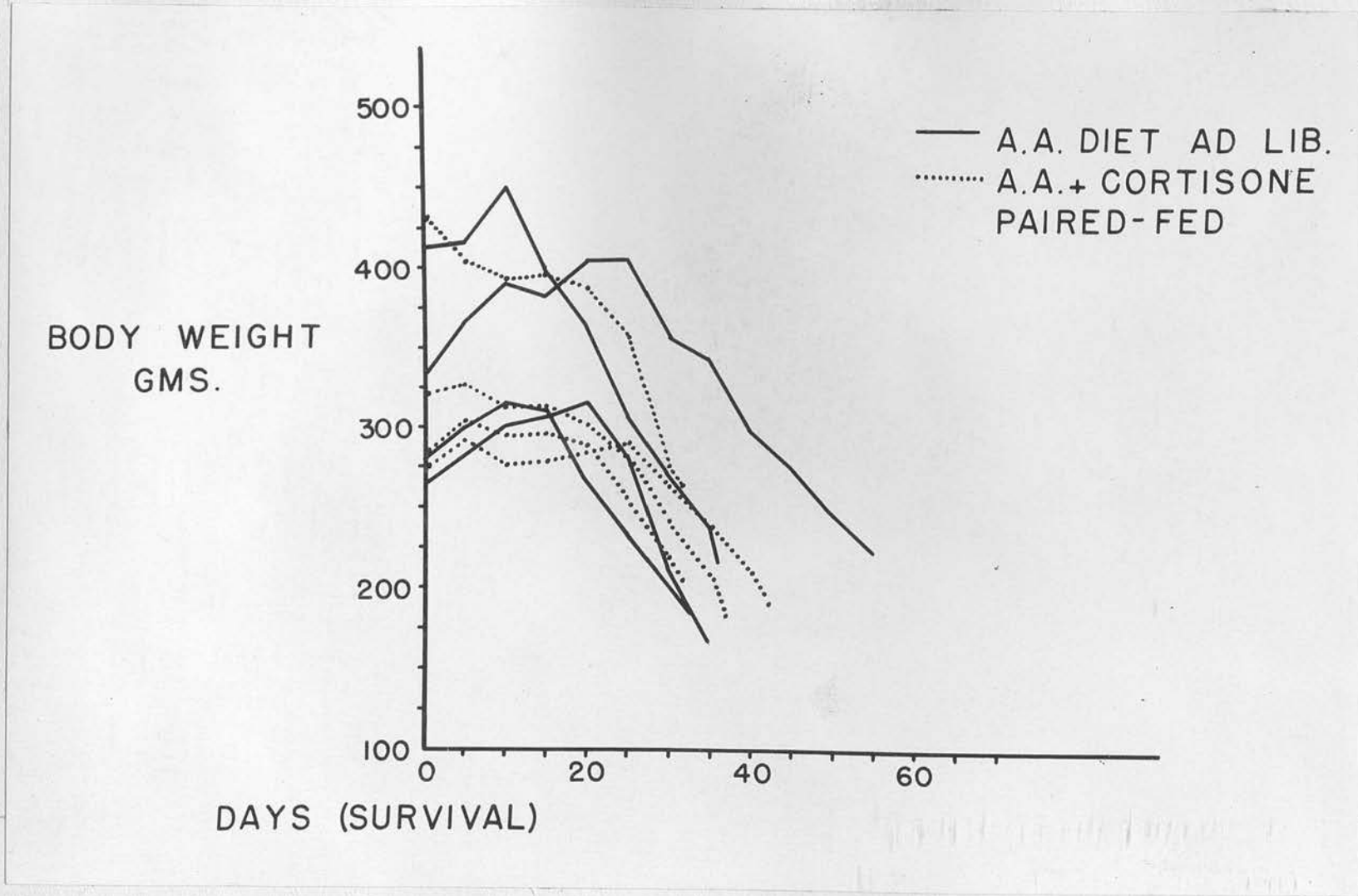


Figure 21. Growth curves of guinea pigs maintained on a scorbutic diet, supplemented with 1 mg. ascorbic acid per os every fourth day. One group was treated with cortisone and paired-fed with the untreated group.

Since these experiments did not confirm the results of Schaffenburg et al (1950) it was decided that the difference might have arisen because he used orange juice as a supplement, and in the experiments now reported pure ascorbic acid was given.

Accordingly, yet a further experiment was performed in which cabbage was used as a source of ascorbic acid, since in this laboratory, it has been the custom to use cabbage as a natural source.

Three groups each containing three male animals were set up as follows:-

Group (1). Scurvy diet and water ad lib and the daily intake was measured; 2 g. cabbage leaf every fourth day.

Group (2). Scurvy diet and water ad lib; 2 g. cabbage leaf every fourth day; daily subcutaneous cortisone in the same dosage as the previous experiment.

Group (3). Like Group (2), but paired - fed with Group (1).

Result.

1). Changes in body weight are given in fig. 22 . They are essentially the same as in the two previous experiments, so that paired - feeding has led to a steady loss of weight, though death ultimately occurs over the same range of time.

2). Survival figures and ratios are as follows:-

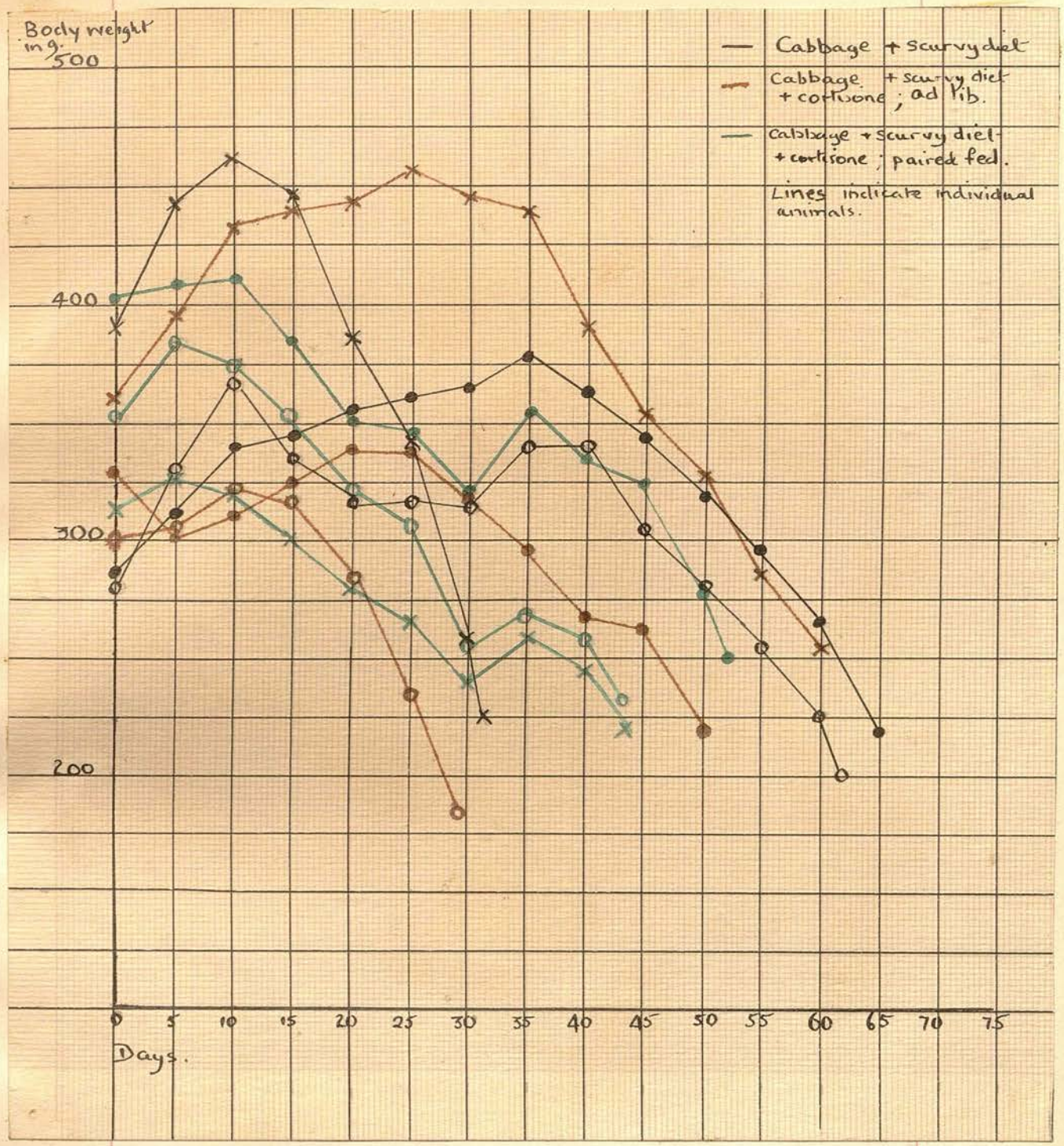


Figure 22. Effect of cortisone with and without paired feeding in guinea pigs receiving 2 g. of cabbage every fourth day.

Table 28.

	Pig	Day of death	Rt. adrenal weight in mg. / Highest body weight in g.
Group (1)	Cabbage only		
	A. Male	65	
	B. Male	32	0.54
	C. Male	62	0.39
Group (2)	Cabbage + cortisone; ad lib feeding.		
	A. Male	50	
	B. Male	60	0.28
	C. Male	28	0.58
Group (3)	Cabbage + cortisone; paired - feeding.		
	A. Male	53	0.15
	B. Male	43	0.15
	C. Male	44	0.22

3). Haemorrhagic manifestations were depressed by cortisone treatment.

4). The mean intake over 10 day periods was as follows:-

	Group 1.		Group 2	
	Food in g.	Water in ml.	Food in g.	Water in ml.
1-10 days	34.6	32.3	24.9	36.4
10-20 days	21.4	24.6	28.7	35.2
20-30 days	20.6	19.1	20.6	27.1
30-40 days	24.6	29.4	17.4	24.6
40-50 days	13.1	31.3	5.1	16.9
50-60 days	10.2	14.6	9.3	23.7
60-70 days	5.1	21.1		

Now the third group which were treated with cortisone and paired - fed with group (1) always ate and drank every scrap of food and water, and were obviously very hungry and thirsty and indeed lost weight. Yet, the intake of group (2) is not really different from that of group (1). It would appear therefore, as though paired-feeding is not very accurate with small groups of animals and group (1) have certainly eaten more than usual, so hiding the differences between groups (1) and (2).

"TOXIC" EFFECT OF CORTISONE.

The effect of cortisone on guinea pigs maintained on a known amount of ascorbic acid has been investigated.

Method.

Four male and four female guinea pigs were placed on a scorbutic diet supplemented with 2 mg. ascorbic acid per os daily. When a steady weight had been reached after twenty days, cortisone was administered subcutaneously in a dosage of 5 mg. daily except on Sundays to two of the four males and two of the females. Body weights were determined daily.

Result.

The body weights are shown in fig. 23.

1). Three out of four of those receiving ascorbic acid only were still alive and well after 115 days, the fourth died of scurvy.

2). The four receiving cortisone in addition, all died between 23 and 52 days. The cause of death was indeterminate; no haemorrhages were present at all and cortisone reduced the right adrenal gland to highest body weight ratio to 0.22.

This result was clear-cut, and has been repeated. It seemed important to determine whether the animals had in fact shown evidence of increased or decreased ascorbic acid requirements and utilization. Accordingly, a further experiment was carried out in order to obtain joint epiphyses for examination.

Method.

A series of male guinea pigs weighing 300-350 g. were placed on a scorbutic diet and treated as follows:-

Group I. cabbage ad lib.

Group II. 2 mg. ascorbic acid (AA) daily per os.

Group III. 2 mg. AA + 1 mg. cortisone acetate subcutaneously.

Group IV. 2 mg. AA + 5 mg. " "

Group V. 2 mg. AA + 10 mg. " "

Group VI. 50mg. AA + 10 mg. " "

Group VII. No treatment.

Treatment was carried on until death occurred, except for Group I which was killed at the end of the experiment. The right adrenal glands were weighed and the knee joints were sectioned.

Result.

1). The survivals and ratios were as follows:-

Group	No. of animals	Mean survival in days	Mean ratio right adrenal gland mg. Highest body wt. mg.
I	4	Remained alive and well	
II	3	1 still alive after 72 days 2 died after 63 and 51 days with pneumonia and pseudo tuberculosis respectively. Mean = 67.	0.30
III	4	66.5	0.28
IV	4	58	0.37
V	4	46	0.19
VI	4	44	0.18
VII	3	23	0.41

Group VII died of typical acute scurvy. The cause of death in the cortisone treated groups is not known, but has occurred earlier as the dose of cortisone has been increased. Cortisone has reduced the adrenal gland, body weight ratio, particularly when the dose was 10.0 mg.

2). In Group II, both at the epiphyses and subperiosteally, there was very definite decreased osteoblastic activity, i.e. 2 mg. ascorbic acid per day led to chronic scurvy in this particular group of guinea pigs. Groups III to V inclusive were grossly abnormal too, but did show some new bone formation both at the epiphyseal lines and subperiosteally, and this was more evident as the dose of cortisone was higher, so that Group V showed more new bone formation than the other groups receiving the same dose of ascorbic acid.

Group VI was approximating to normal. I am indebted to Dr. H. Spencer for help in interpreting these slides.

It does appear therefore, as though cortisone has stimulated new bone formation. The cause of death from the "toxic" effect of cortisone however, remains unsolved but there is no evidence to suggest that it is due to scurvy.

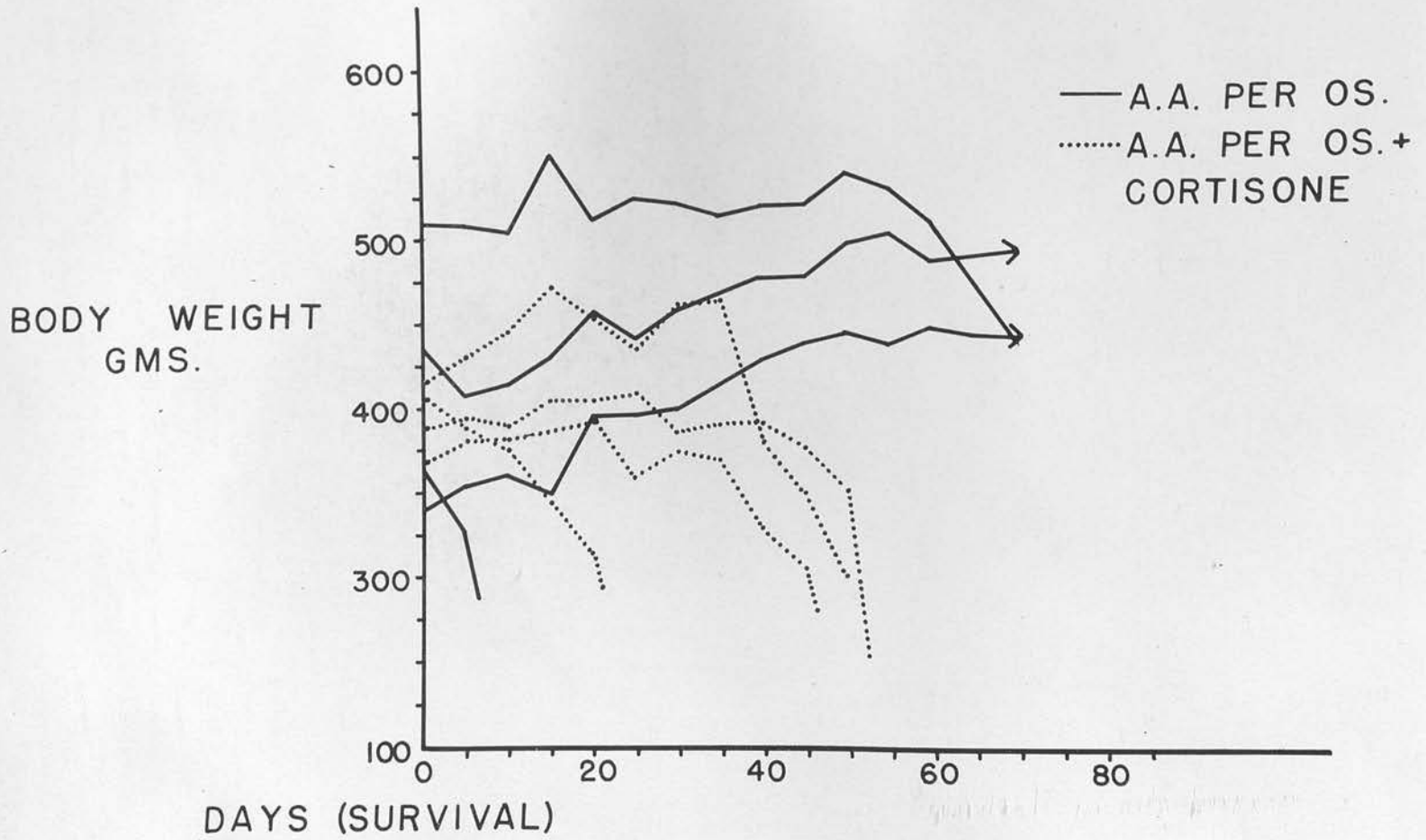


Figure 23 . Effect of 5 mg. cortisone daily on the survival of guinea pigs maintained on a scorbutic diet supplemented with 2 mg. ascorbic acid daily per os.

ADRENALECTOMY IN GUINEA PIGS.

EXPERIMENTAL

Technique of adrenalectomy in the guinea pig.

The mortality immediately following adrenalectomy is high and about one third ultimately survive the complete operation, though on occasion it has been as low as 10%.

Daily administration of 2 mg. DOCA-in-oil subcutaneously is begun seven to ten days before operation, and is continued throughout the rest of the animal's life. The right gland is removed first, followed by removal of the left one week later.

Fifteen minutes before operation 1/100 gr. atropine is given intraperitoneally, since the guinea pig has a narrow trachea which easily becomes blocked with secretion. Operation is performed under ether anaesthesia, induction being carried out in a closed vessel, and anaesthesia completed on the table by means of ether on cotton wool. (Cotton wool makes ether become toxic very rapidly, so it should be changed frequently in all animal work).

During anaesthesia the animals pass through a stage when they show "athetoid-like" movements. Operation should not begin until this stage is past.

The hair is soaked with spirit but not shaved off. A dorsal incision is made through skin and muscle and the last rib. The glands are removed by gentle blunt dissection. Removal of the right one is technically rather difficult owing to its close proximity to the renal vessels and the

inferior vena cava. It is not necessary to tie off any vessels. Any bleeding is usually controlled by pressure, though if severe, clotting can be encouraged with gelatin sponge. If sponge is used on the right side, adhesions make removal of the left gland more difficult, so its use should be avoided when possible. The muscle and skin are closed in layers with interrupted silk ligatures.

Completely adrenalectomised animals are kept at 70°F and fed especially on cabbage ad lib, and some bread and milk, Rowett cubes and water or 1% sodium chloride. On this regime they survive indefinitely and are used for most experiments two weeks after operation has been completed.

The Response of Adrenalectomized Guinea Pigs to ACTH.

Method.

Groups of four male and four female adrenalectomized guinea pigs were placed in pairs in metabolism cages and maintained on a scorbutic diet supplemented with cabbage ad lib. In each group, one pair received ACTH and the other distilled water, using the same dosage and regime as described in the experiment on the response of late scorbutic animals to ACTH. Urinary 17-ketosteroids were determined before treatment, on each day of treatment and for several days thereafter.

Result.

The 17-ketosteroid excretion is given in Table 29. The injection of ACTH or distilled water caused no change at all in the already negligible excretion of 17-ketosteroids, and there was no change in body weight.

TABLE 29.

RESPONSE OF ADRENALECTOMISED GUINEA PIGS TO ACTH.*

Urinary 17 ketosteroid excretion mg./day/guinea pig.

	Experiment 1. (Male)		Experiment 2 (Female)	
	ACTH	Distilled water	ACTH	Distilled water
Before treatment	0.19	0.06	0.09	0.07
	0.10	0.10	0.07	0.01
	0.15	0.10	0.13	0.08
During treatment				
Day 1	0.11	0.10	0.10	0.16
2	0.18	0.18	0.14	0.12
3	0.14	0.17	0.14	0.12
4	0.13	0.26	0.14	0.13
After treatment	0.20	0.19	0.17	0.08
	0.13	0.14	0.12	0.09
	0.10	0.18	0.12	0.09

* All animals were receiving 2 mg. DOCA daily.

The Effect of Progressively Reducing the Dose of DOCA in Adrenalectomized Animals.

The daily dose of DOCA in two adrenalectomized male guinea pigs was progressively diminished, and the effect on 17-ketosteroids was examined. Details of dosage and results were as follows:-

Days since operation was completed.	Dose of DOCA in mg.	17-ketosteroids in mg/24 hours/animal.
1 to 18	2 mg.	Day 8 0.075 Day 12 0.150 Day 16 0.250 Day 19 0.278
19 to 26	1.5 mg.	Day 25 0.103
27 to 33	1 mg.	Day 32 0.138
34 to 40	0.5 mg.	Day 29 0.152
41 to 68	nil	Day 47 0.030 Day 57 0.135 Day 64 0.139

DOCA-in-oil is probably accumulative, and this would account for the survival after DOCA administration had ceased. The excretion of "17-ketosteroids" has remained much the same and it is doubtful that this is true steroidal material in any case.

The Effect of Suddenly Stopping DOCA in Adrenalectomized Guinea Pigs.

Method.

The administration of 2 mg. daily DOCA to two adrenalectomized males was stopped thirty three days post-operatively.

Result.

The animals survived for a further fifteen days, probably because DOCA in oil is cumulative.

The 17-ketosteroid level on DOCA was 0.105 mg/24 hours/animal & was 0.112, 0.033, 0.093 in the 6th, 13th and 14th days after DOCA ceased.

Dietary Intake of Adrenalectomised Animals.

During the experiment on the gradual reduction of the dose of DOCA in adrenalectomized guinea pigs, the intake of cabbage and scurvy diet has been measured, and the sodium content of the diet on each day has been calculated. The sodium content of scurvy diet is as follows:-

oats: 4.4 mg%; bran: 14.0 mg%; egg: 519.0 mg%;

salt: 39310.0 mg%; cabbage: 57.5 mg%.

The daily intake is shown in detail in fig. 23. It will be seen that the daily intake of sodium lies between 150 and 300 mg. usually. The most striking feature is the enormous amount of cabbage which is eaten - this is a very striking feature of some adrenalectomized guinea pigs, as an intact animal will only eat about 70 g. a day or less over a length of time.

The dietary intake in the experiment in which the administration of DOCA was completely stopped, is shown in fig.24. In this case, cessation has been followed by a sharp rise in the intake of scurvy diet, with a consequent rise in the sodium intake. This intake was not maintained and the animals subsequently died; the intake remained

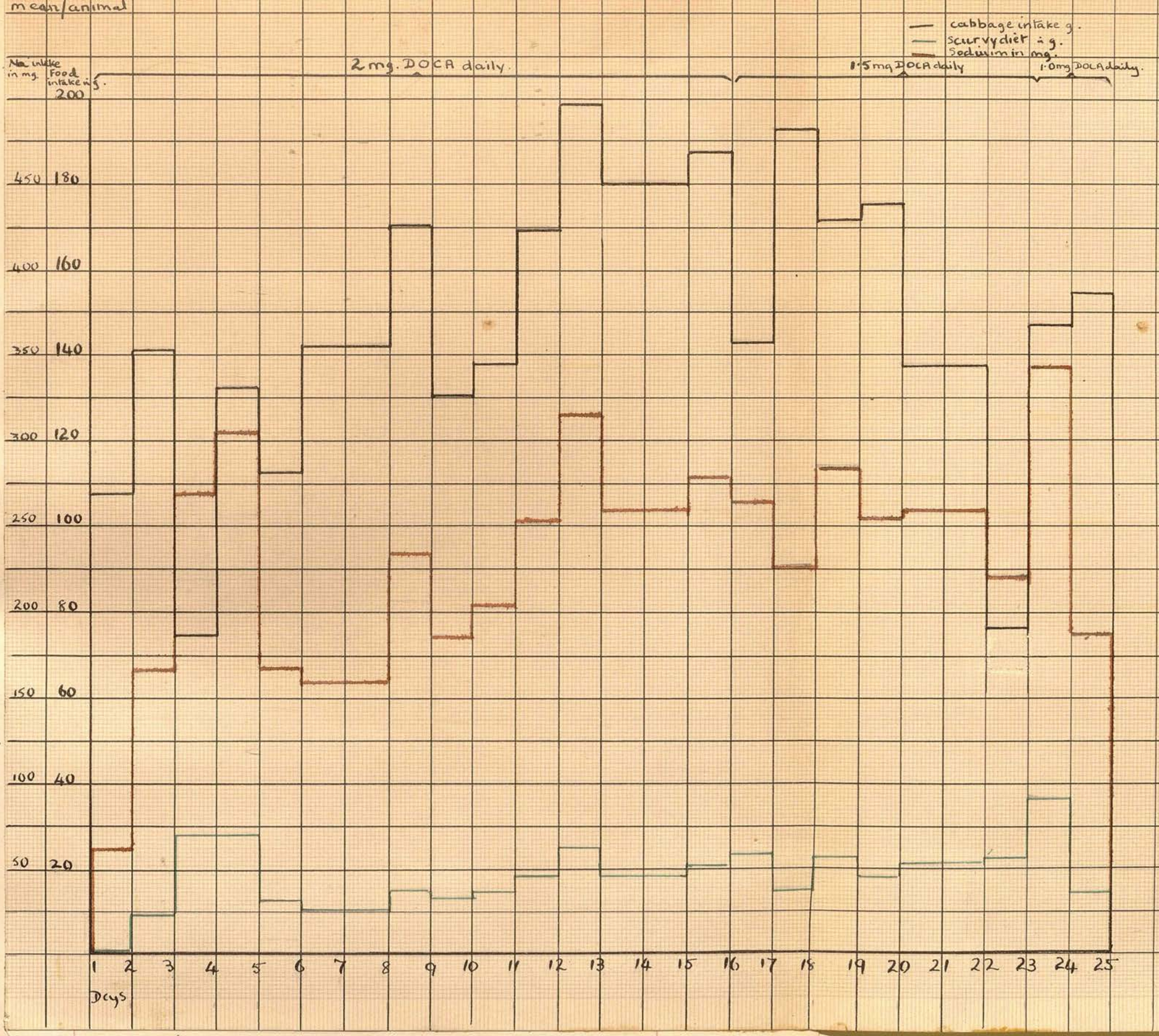


Figure 23 . Food intake chart; effect of gradually reducing the dose of DOCA in male adrenalectomized guinea pigs.

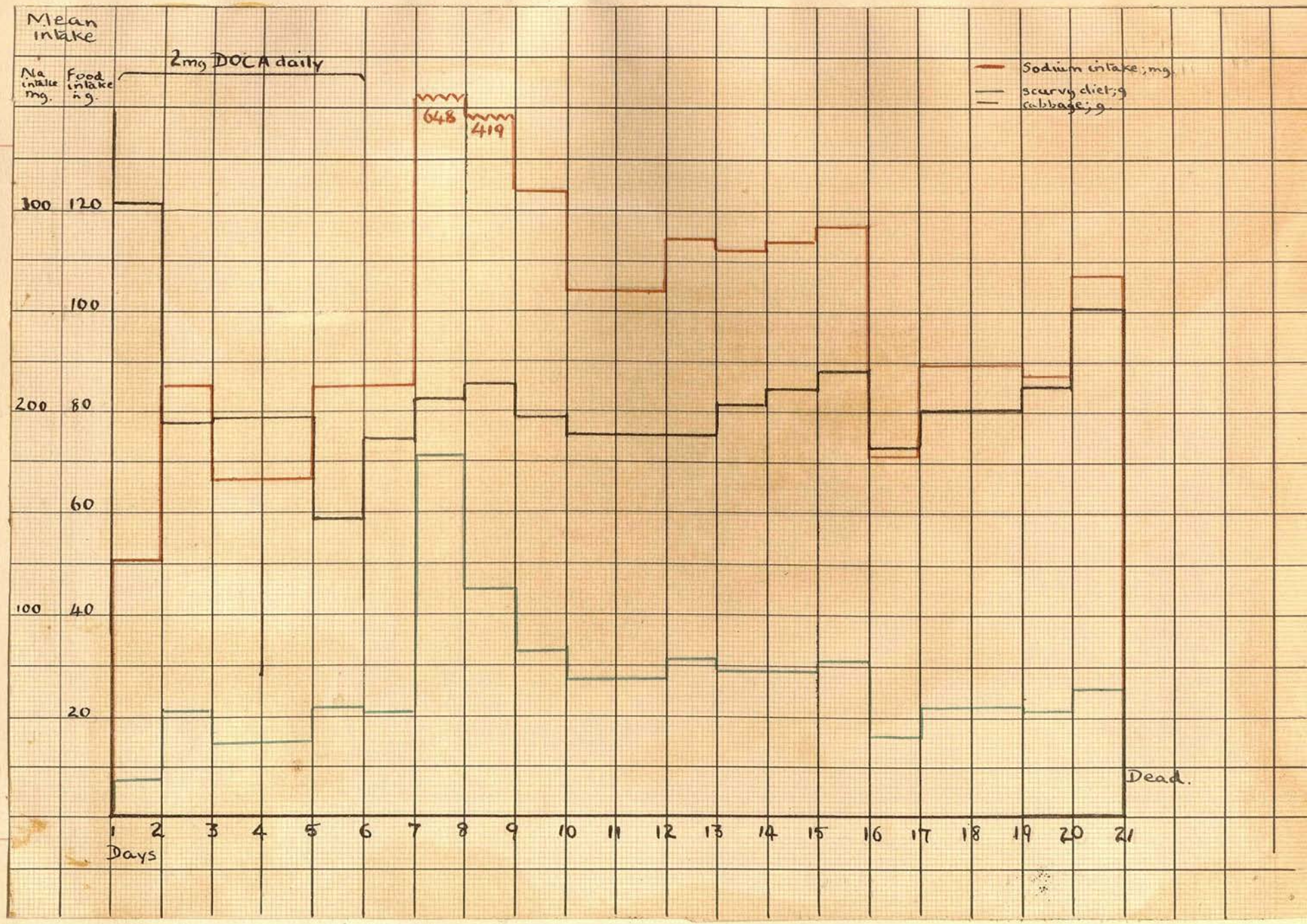


Figure 24. Food intake chart. Effect of stopping DOCA administration in a male adrenalectomised guinea pig.

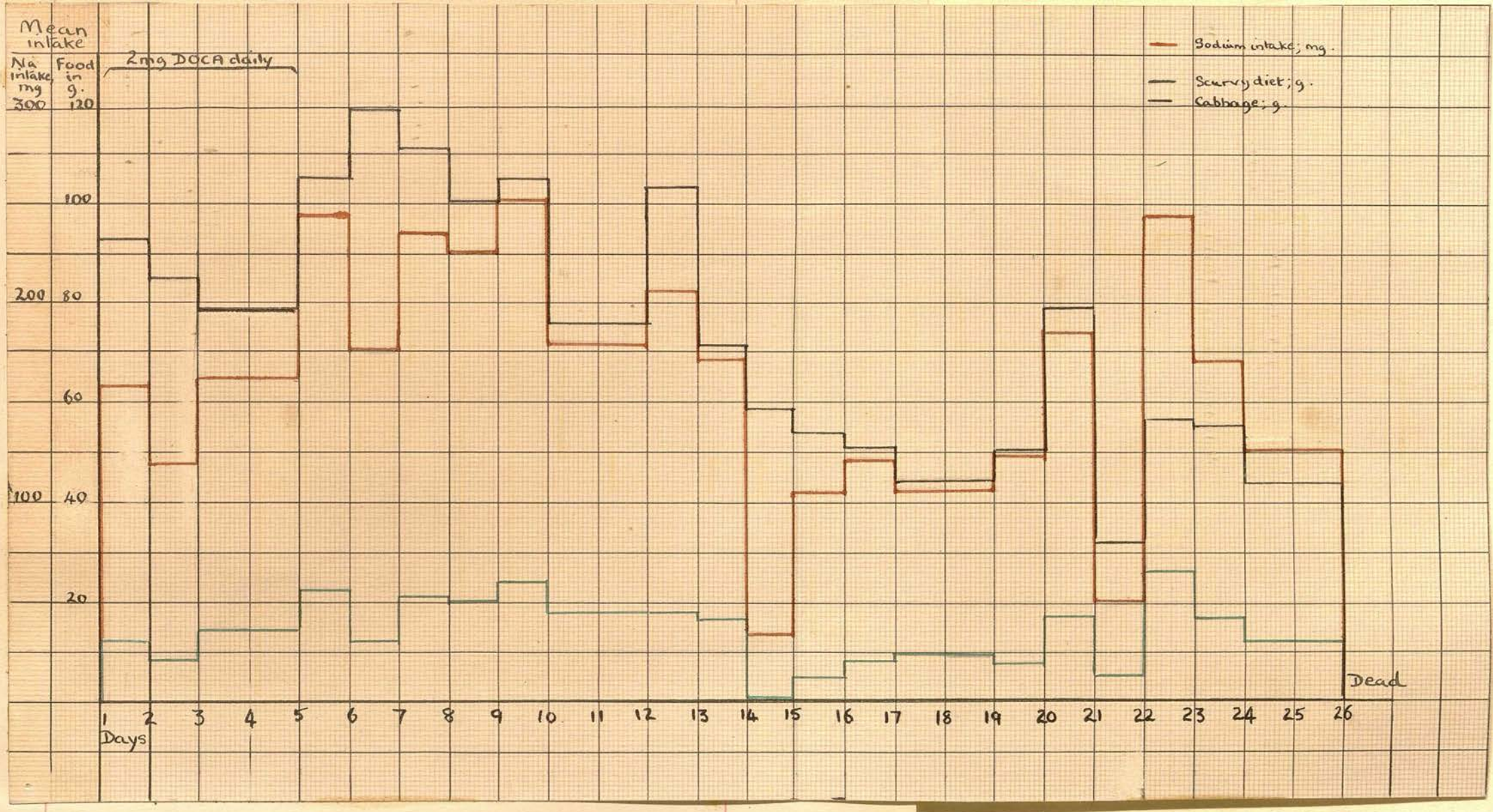


Figure 25. Food intake chart.

Effect of stopping DOCA administration in a female adrenal-ectomised guinea pig.

surprisingly high, however, up to the time of death. Another pair of animals, however, responded by increasing their cabbage intake, though this does not increase the sodium intake very much (see fig. 25).

In the experiment in which ACTH or distilled water was administered to adrenalectomised animals, dietary intake was measured. During and following treatment with ACTH and water, the males showed a large increase in their cabbage intakes (figs. 26 and 27), and this was apparent in the females too following treatment with water but not ACTH. It appears as though adrenalectomised animals respond to a stress (in this case injection) by increasing their cabbage intake, and occasionally their scurvy diet (and hence sodium) intake too. Cabbage appears to exert a specific effect on adrenalectomised guinea pigs since it has been found impossible to keep them alive without it, even though very large amounts of lettuce and celery have been supplied instead. Also, when cabbage has been replaced by daily ascorbic acid by mouth (in dosage 10 mg/100 g. body weight) the animals have lost weight and not survived more than ten days.

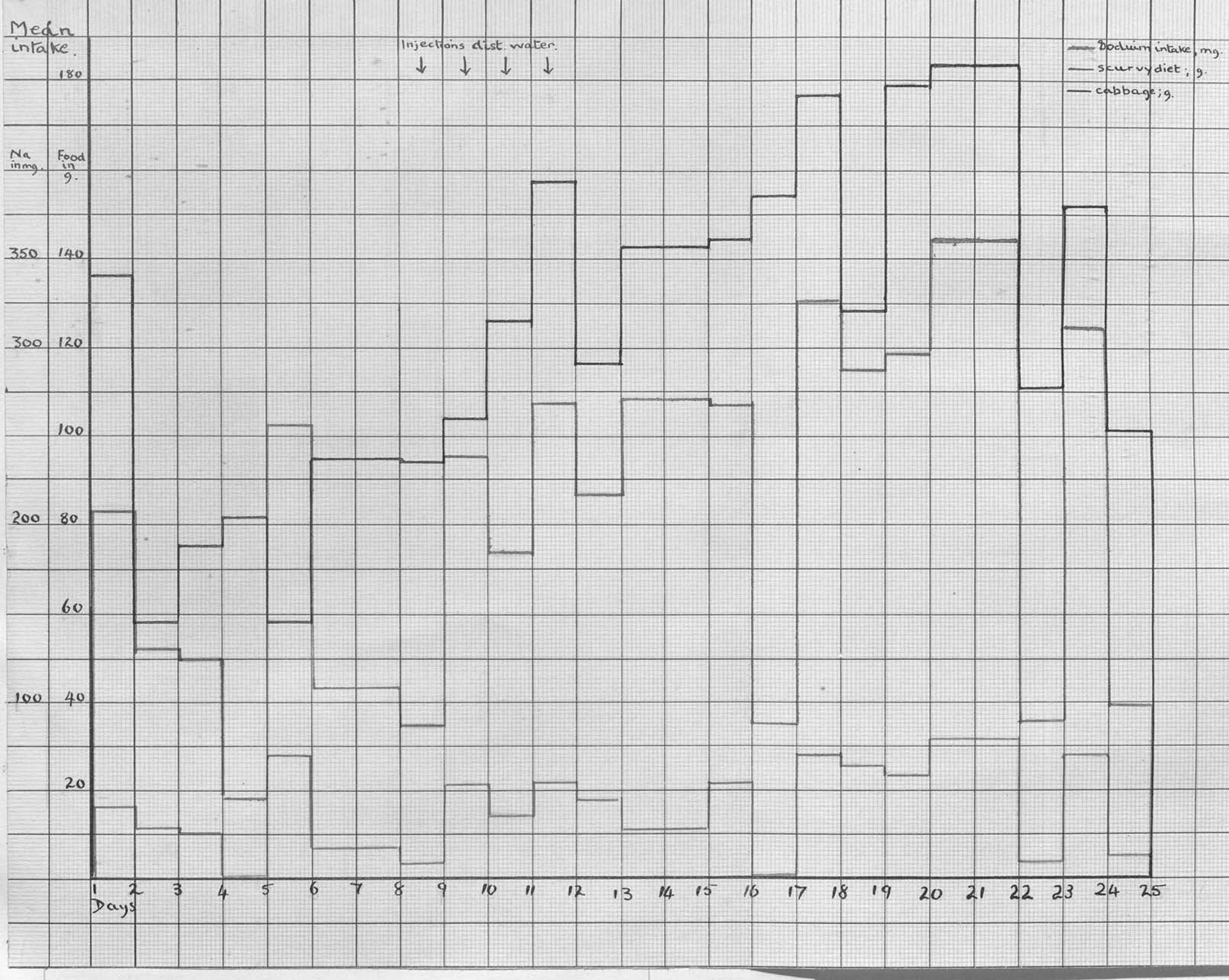


Figure 26. Food intake chart.

Effect of injecting distilled water on four consecutive days to adrenalectomised male guinea pigs.

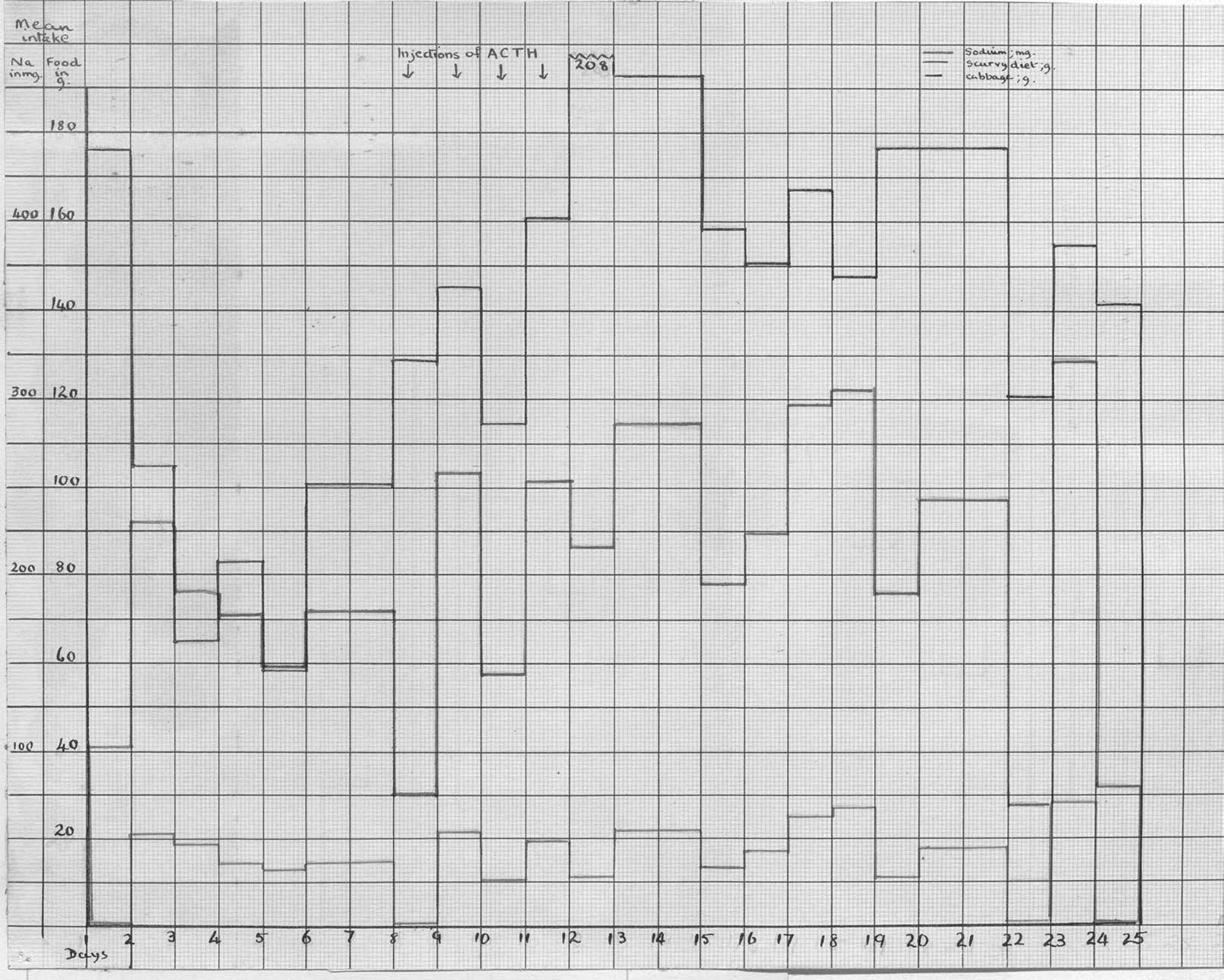


Figure 27. Food intake chart.

Effect of injecting ACTH on four consecutive days to adrenal-ectomised male guinea pigs.

GONADOTROPHINS AND 17-KETOSTEROIDS.

EXPERIMENTAL.

EFFECT OF GONADOTROPHINS ON 17-KETOSTEROID EXCRETION IN CASTRATED GUINEA PIGS.

Method.

The gonads were removed from a series of guinea pigs, weighing 400 - 500 g. Ovaries were removed through a dorsal incision and testes through the upper part of the scrotum. Treatment was begun two weeks after operation. Pairs of guinea pigs were grouped as follows:-

Group (1). female; daily subcutaneous injection of a suspension of dried egg in water.

Group (2). male; ditto.

Group (3). female; 50 I.U. chorionic gonadotrophin (C.G.) daily subcutaneously in 0.5 ml. water.

Group (4). male; ditto.

Group (5). female; 50 I.U. pregnant mares' serum (P.M.S.) gonadotrophin daily subcutaneously in 0.5 ml. water.

Group (6). male; ditto.

Periodic 17-ketosteroid determinations were carried out; all animals were examined when the experiment was terminated. Chorionic and pregnant mares' serum gonadotrophin were also injected into pairs of intact animals, but 17-ketosteroids were not determined.

Result.

1). Urinary 17-ketosteroids in mg/day/animal.

	Group (1) (egg-yolk)	Group (3) (chorionic gonadotrophin)
Females		
Pretreatment	0.176	0.231
excretion.	0.176	0.159
	0.180	0.140
Days after beginning treatment.		
5	not done	0.082
12	0.180	0.121
15	0.154	0.113
26	0.258	0.125
33	0.241	0.193
36	0.212	0.160
40	0.217	0.187
43	not done	0.206

	Group (2) (egg-yolk)	Group (4) (chorionic gonadotrophin)
Males		
Pretreatment	0.158	0.247
excretion	0.176	0.135
		0.129
Days after beginning treatment.		
5		0.177
12		0.093
15	0.142	0.086
26		0.167
33	0.287	0.170
36	0.310	0.147
40	0.264	0.210
43	0.188	0.210

	Group (5) P.M.S. gonadotrophin)	Group (6) P.M.S. gonadotrophin)
Females		
Pretreatment	0.145	0.172
excretion	0.247	0.203
Days after beginning treatment.		
35	0.152	0.133
42	0.187	0.220
49	0.220	0.245
56	0.183	
59		0.208

There is no response by the adrenal glands, as shown by 17-ketosteroid excretion, to injected chorionic or pregnant mares' serum gonadotrophin, in the castrated guinea pig.

2). Both gonadotrophins produced typical macroscopic changes in the intact animals. Castrated animals treated with egg yolk in gonadotrophin showed atrophy of the generative organs. Chorionic gonadotrophin, but not pregnant mares' serum gonadotrophin, resulted in the loss of much fur, and an almost, complete disappearance of subcutaneous and abdominal fat in castrated male and female animals - this was a very striking and unexpected feature. Microscopic examination of the ovaries of C.G. treated animals revealed a large increase in the amount of ovarian stroma, and a very little luteinization. P.M.S. caused some increase in the number of follicles. The ovarian changes, however, were not as striking as one would wish.

3). The adrenal weights in mg. were as follows:-

Intact, saline treated females	155.0	224.0
Intact P.M.S. treated females	238.0	224.0
Intact C.G. treated females	250.9	215.7
Ovariectomised egg-yolk treated females	183.4	143.2
Ovariectomised P.M.S. treated females	167.0	230.0
Ovariectomised C.G. treated females	115.3	150.8
Gonadectomised egg-yolk treated males	184.4	260.9
Gonadectomised P.M.S. treated males	303.0	192.0
Gonadectomised C.G. treated males	160.5	138.0

The zona glomerulosa of the adrenal of ovariectomized animals was increased in thickness, and even more so in those treated

with C.G., but not with P.M.S. Ovariectomised C.G. treated animals also showed a reduced amount of fat in the zona glomerulosa and foscuiculata. This was not apparent in the male animals.

ACTH ADMINISTRATION TO PREGNANT GUINEA PIGS.

In the course of some work which is still being carried on, 20 I.U. ACTH B.I.D. (Armour gel L.2008) was administered subcutaneously to twenty one pregnant guinea pigs near term for seven days. The animals remained in good health and did not abort.

17-ketosteroids were determined on the urines of two. In the first the excretion prior to ACTH was 0.44 mg/24 hours, and on the 5th, 6th and 7th days of injection was 1.95, 1.95 and 2.44 mg/24 hours respectively, and in the second the baseline excretion was 0.62, and on the 3rd and 4th days of injection 2.7 and 3.3 mg/24 hours respectively. These are the highest excretions obtained in the guinea pig experiments so far. They suggest that the adrenals of the pregnant animals are very sensitive to ACTH - possibly from the action of pregnancy gonadotrophin. This line of work will be pursued further.

The adrenal glands in the two ACTH-treated pregnant animals were particularly large, but showed no hyperaemia or haemorrhage.

Weight right adrenal in mg.

Untreated, pregnant

F	226.0
G	290.5

ACTH treated, pregnant

E	705.1
D	414.0

It is interesting to compare the size of the foetal adrenal glands in these four animals:-

Weight of left adrenal in mg.

F	10.0	11.5	9.0	
G	9.5	12.0	10.0	
E	4.5	5.0	3.5	
D	4.3	8.0	2.6	3.9

Those of the ACTH treated animals are much smaller, suggesting that corticoids but not ACTH have crossed the placenta. The foetuses showed no obvious abnormalities.

EFFECT OF CORTISONE ON APPETITE WITH SPECIAL REFERENCE TO CABBAGE.

EXPERIMENTAL.

EFFECT OF CORTISONE IN THE GUINEA PIG.

The effect of increasing amounts of cortisone on the appetite of guinea pigs maintained on the scurvy diet supplemented with cabbage and ascorbic acid has been examined, as I feel that cabbage may exert a protective effect on the animal against cortisone.

Method.

Pairs of male guinea pigs weighing 300 g. were treated with increasing amounts of subcutaneous cortisone (1.25 to 25 mg) following a baseline period. Their diets were:-

Group (1). Scurvy diet ad lib + 42.5 g. cabbage 1 pig + tap water ad lib.

Group (2). Scurvy diet ad lib + 25 mg. ascorbic acid per os in water + tap water ad lib.

Body weights were determined daily.

Result.

The body weights and food intake are shown in fig. 28 in detail. Though Group (2) never gained so well as Group (1), their weight curves were rising until cortisone treatment began, and then both of them eventually died; both animals appeared healthy at death and their right adrenal glands were depressed to 81 and 100 mg. as compared with 139 and 125 mg. in Group (1).

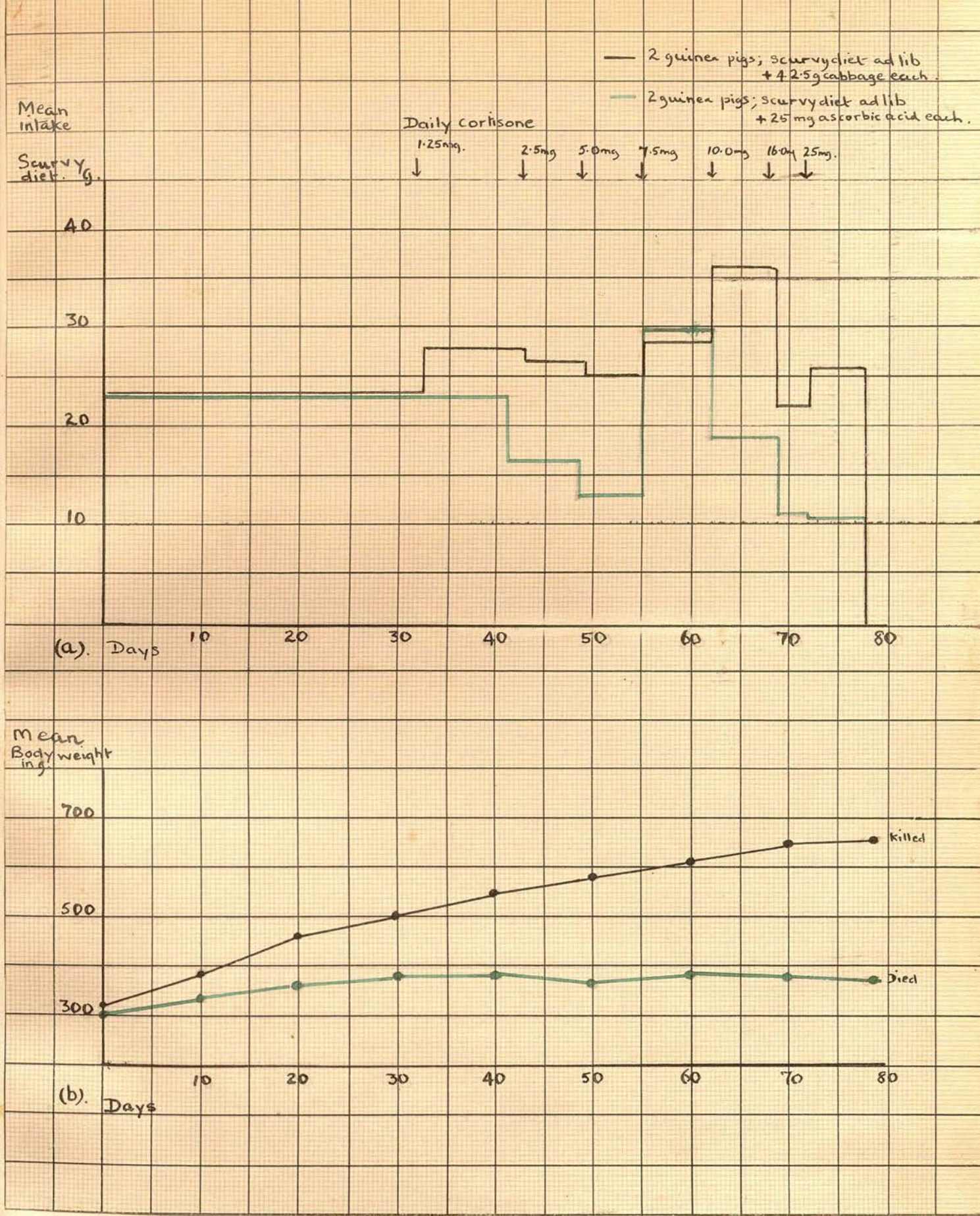


Figure 28 . Effect of cortisone on pairs of guinea pigs maintained on scurvy diet supplemented with ascorbic acid and cabbage.

a). Mean food intake.

b). Mean body weight at 10 day intervals.

In those receiving cabbage, cortisone immediately began to increase the appetite, particularly when the dosage was increased to 10.0 mg. The effect in Group (2) is obscured by the pig dying early in the experiment, but the appetite is higher over the period of 55-62 days and when the dose reached 10 mg. it began to fall rapidly. It does appear as though cabbage in the diet has enabled the animal to withstand the "toxic" effect of cortisone.

THE EFFECT OF CORTISONE IN THE RABBIT.

From the literature it appears that the rabbit is far more sensitive to cortisone than a guinea pig (Ragan et al (1949)). A large male rabbit weighing about 5 lbs. was placed on a measured ad lib diet of cubes + cabbage and water. After a baseline period, it was given 5 mg. cortisone subcutaneously per day, increased to 10 mg. a day after three weeks. Eight days later treatment was stopped.

Result.

The intake of everything rose sharply as soon as cortisone was begun,but no further rise occurred when the dose was increased. The appetite fell rapidly after stopping cortisone and the animal died in three days. Full details are shown in Fig. 29.

The response has been very rapid with a dose which is very small in comparison with the size of the animal. The rabbit presumably died of adrenal failure following the sudden cessation of cortisone.

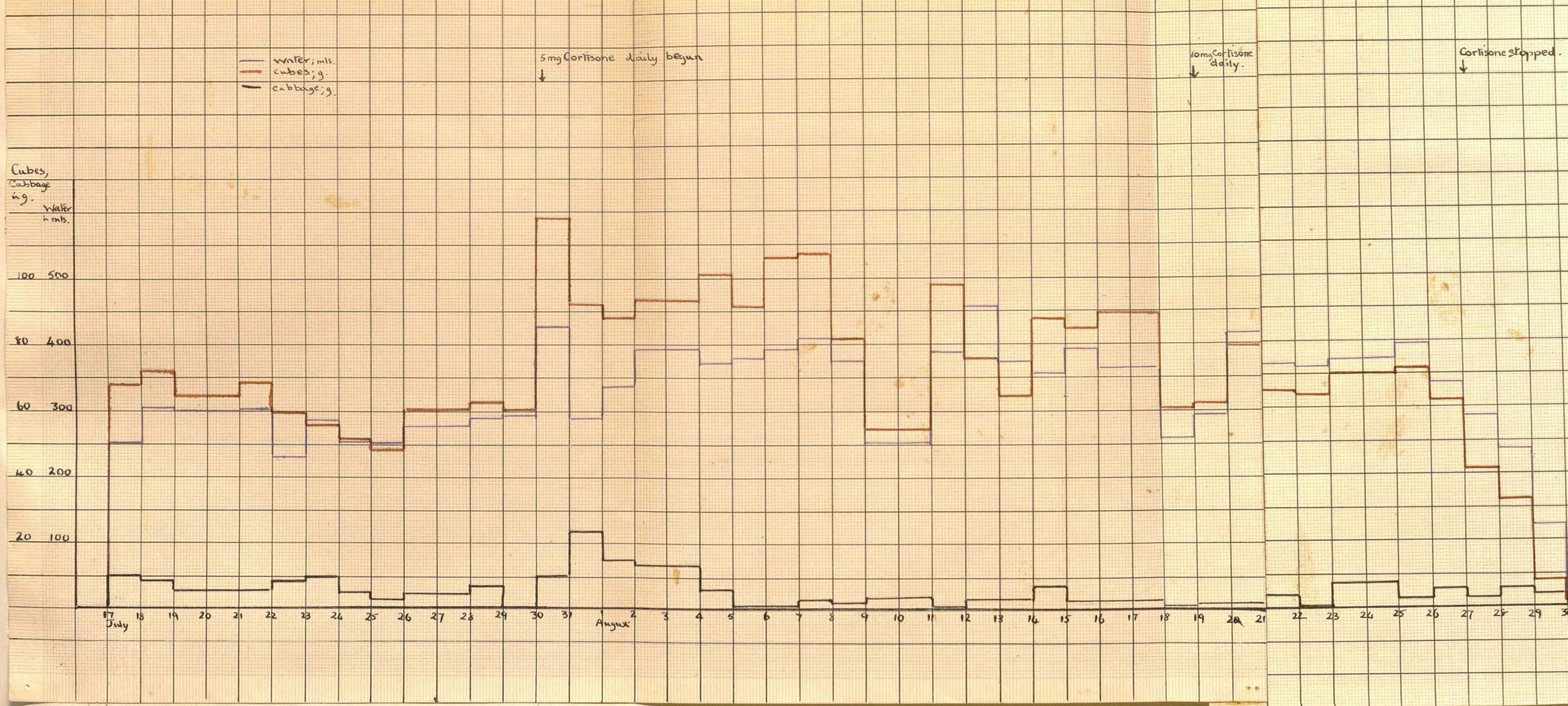


Figure 29. Effect of cortisone on the food intake of a male rabbit maintained on Rowett cubes, cabbage and water.

TOXICITY AND ANTISCORBUTIC EFFECT OF DEHYDROASCORBIC ACID.

EXPERIMENTAL.

Two preparations of dehydroascorbic acid (DHA) were available:-

1). A preparation prepared by Boots Ltd. This was 83% pure and was a brownish - yellow powder. It was made up freshly before use by adding water and holding the container in boiling water for about half a minute to get it into solution, and then cooling rapidly in ice, and solutions were always used immediately. The dry powder was stored in a dessicator after an ampoule was opened.

2). A preparation of dehydroascorbic methanolate prepared by Roche Ltd., which was 80% pure. It was a white powder which was readily soluble in cold water. The powder was stored in the refrigerator in approximately 1 gm. ampoules, and solutions were used immediately.

TO INVESTIGATE THE ANTISCORBUTIC EFFECT OF DEHYDRO-ASCORBIC ACID (BOOTS).

Methods.

Thirteen male guinea pigs weighing about 300 g. were placed on a scurvy diet, supplemented with cabbage for 21 days. Body weights were determined daily. The cabbage supplement was then withdrawn and when the weight of an animal began

to fall (after 14 - 18 days) treatment was begun. The animals were treated as follows:-

a) 3 guinea pigs - daily ascorbic acid per os.

b) 4 guinea pigs - daily DHA per os.

c) 4 guinea pigs - daily DHA by subcutaneous injection

d) 2 guinea pigs - no treatment.

Ascorbic acid or DHA was administered once daily in a dosage of 20 mg. per animal, increased to 60 mg. after 17 to 21 days.

At autopsy, the right adrenal gland was weighed. Moribund animals were killed with ether and the ascorbic acid content of the right adrenal gland was determined. Appropriate tissues were sectioned.

Result.

1). Untreated animals died of typical acute scurvy.

2). Ascorbic acid treated animals gained weight. One died after three months with a severe sore on its chin, and one after five and a half months with pneumonia. The third was still alive and well after nine months. None of these animals showed any features of scurvy, and all had healthy livers. The ratio of right adrenal gland to body weight was increased in the pneumonia guinea pig but not in the other two.

3). All those treated with DHA ultimately died, and all of them showed grossly fatty livers. A rise in body weight occurred initially and then the weights began to show large fluctuations (see fig. 30).

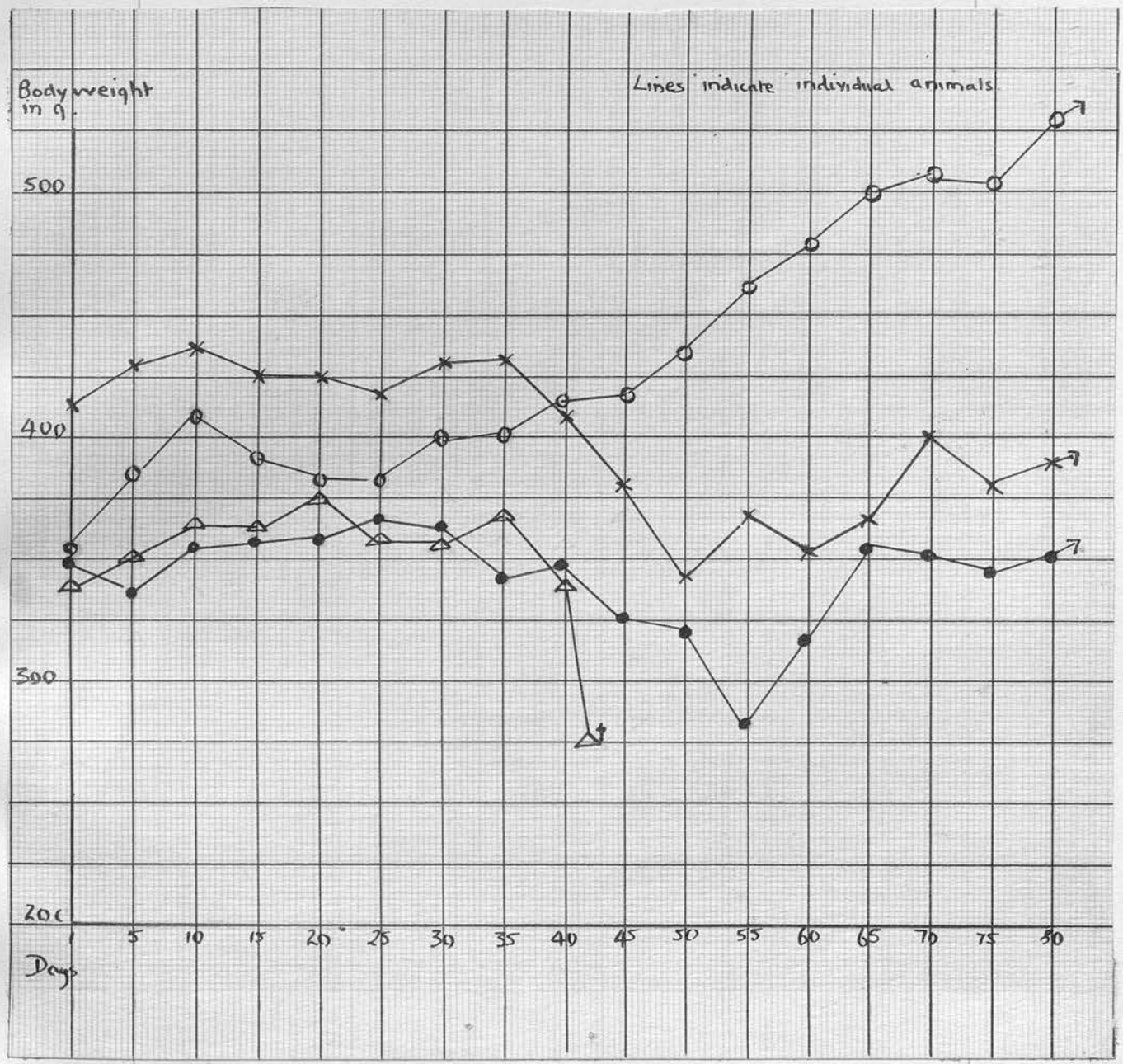


Figure 30 . Effect of 60 mg. DHA (Boots) per os on body weights of male guinea pigs on a scorbutic diet.

Two to three weeks before death, these animals began to lose their fur, particularly over the terminal phalanges of the fore and hind feet, and the remaining hair became starring. The digits became very flexed. A day or two before death, respiratory difficulty developed, and there was often partial or complete paralysis of the hind legs (see fig. 31). A Benedict's test for sugar was performed on the urine of one DHA treated animal and gave a negative result.

Three out of eight DHA - treated animals had high right adrenal gland to body weight ratios, though no haemorrhages were present, and indeed the adrenal ascorbic acid content determined in one of these was quite high (see Table 30).

Microscopic Examination of the Tissues in DHA - treated Animals.

a). Livers - gross fatty degeneration, especially at the centres of the lobules.

b). Joints - the orderly arrangement of the cells at the epiphyses was maintained, and there was no evidence of scurvy. There were no haemorrhages, but the number of fat cells in the marrow was increased.

c). Kidneys - there was hyaline degeneration of the glomeruli, occasionally complete. A few epithelial crescents were present. Some tubules contained hyaline casts.

Figure 31. The effect of dehydroascorbic acid (Boots) on guinea pigs.

(a). Male guinea pig which has received scurvy diet, supplemented by 5 mg. ascorbic acid daily per os. The fur is sleek, eyes are bright and the animal is well - nourished.

(b). Male guinea pig which has received 60 mg. dehydroascorbic acid daily per os.

(c). Male guinea pig which has received 60 mg. dehydroascorbic acid daily by injection.

Fore feet of guinea pig shown in (c).

Hind feet of guinea pig shown in (c).

d). Testes - there was inhibition of spermatogenesis.

e). Pancreas - the islets were probably reduced in size and number, though this was difficult to assess with any certainty.

Detailed results of ratios and ascorbic acid content of adrenal glands are given in the next table. 30.

Table 30.

Guinea pig	Treatment	Survival in months after withdrawal of cabbage	Highest body weight obtained in g.	Ratio rt. adrenal gland in mg. / Highest body weight in g.	Ascorbic acid content of rt. adrenal gland in mg/100 g.	Remarks
1	None	26 days				
2	None	34 days				
3	Ascorbic acid per os.	5½	620	0.54		Pneumonia
4	"	9	570	0.34	20.4	
5	"	3	460	0.37		Sore on chin
6	DHA per os	3½	380	0.36	12.6	
7	"	6	465	0.37		
8	"	8	685	0.24	11.0	
9	"	1½	379	0.41		
10	DHA injection	2	450	0.26	26.1	
11	"	4	375	0.48	44.6	
12	"	1½	400	0.34		
13	"	5	590	0.44		

TO EXAMINE THE ANTISCORBUTIC EFFECT OF DEHYDROASCORBIC ACID (ROCHE).

Method.

A series of six male guinea pigs weighing about 350 g. were placed on a scorbutic diet supplemented with cabbage for ten days. The cabbage was then stopped and the animals were depleted for twenty days. Dehydroascorbic acid (Roche) was then begun; three received the equivalent of 60 mg/day by injection and three per os. Body weights were determined daily.

Result.

1). Fig.32,33 shows the body weight curves at five-day intervals.

2). Survival and adrenal to body weight ratios follow:-

	Survival in days.	Right adrenal gland in mg. / Highest body weight in g.
DHA per os		
B	59	0.40 *
D	52	0.43
F	108 (killed)	0.32
DHA by injection		
A	24	0.36
C	10	0.39
H	62	0.37

* When B was moribund the urine was tested for sugar and the result was negative.

Survival following DHA by injection has been poor though an antiscorbutic effect has definitely been seen in pig H. Following DHA per os there has been a large gain in weight,

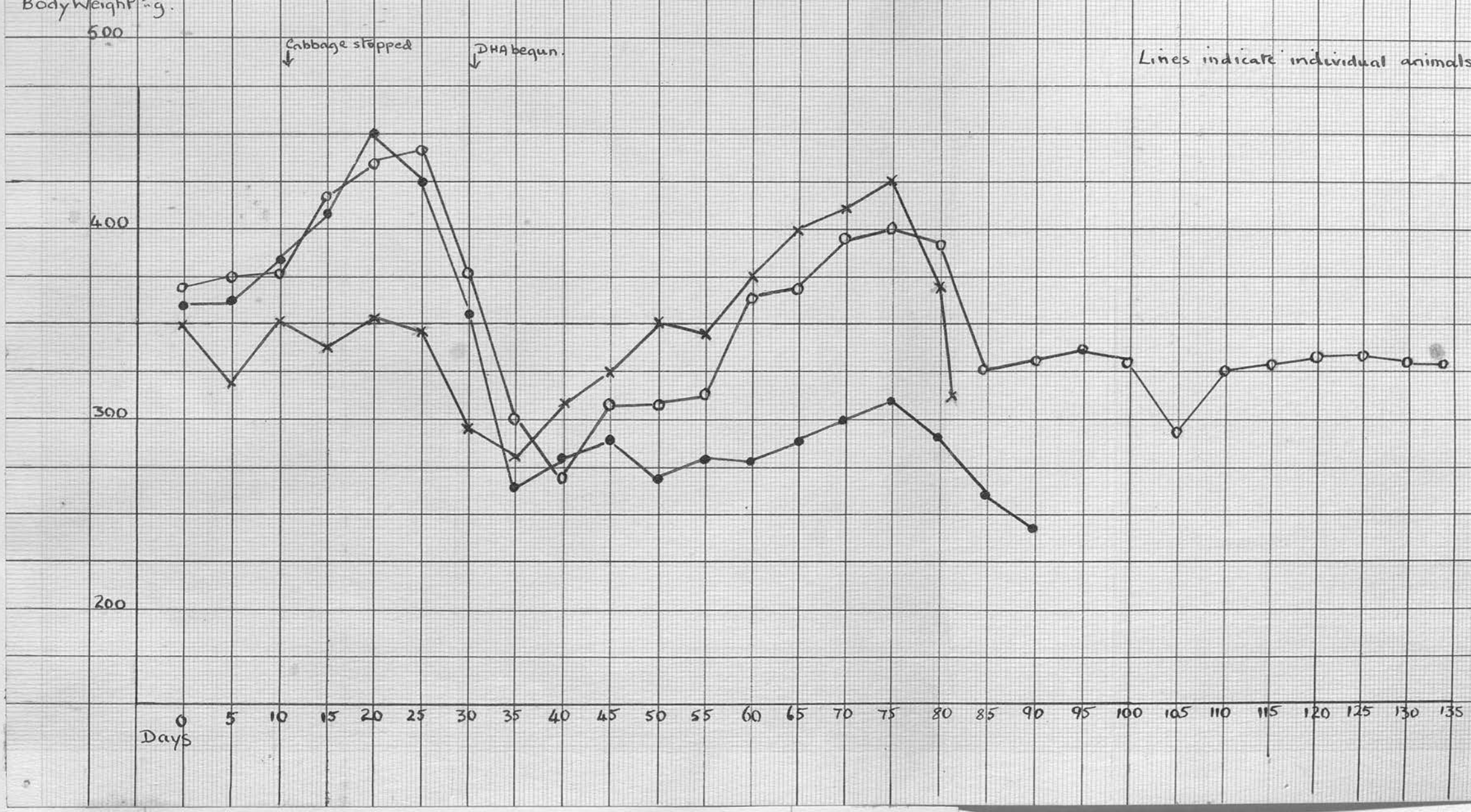


Figure 32 . Effect of 60 mg. DHA (Roche) per os on the body weights of male guinea pigs maintained on a scorbutic diet.

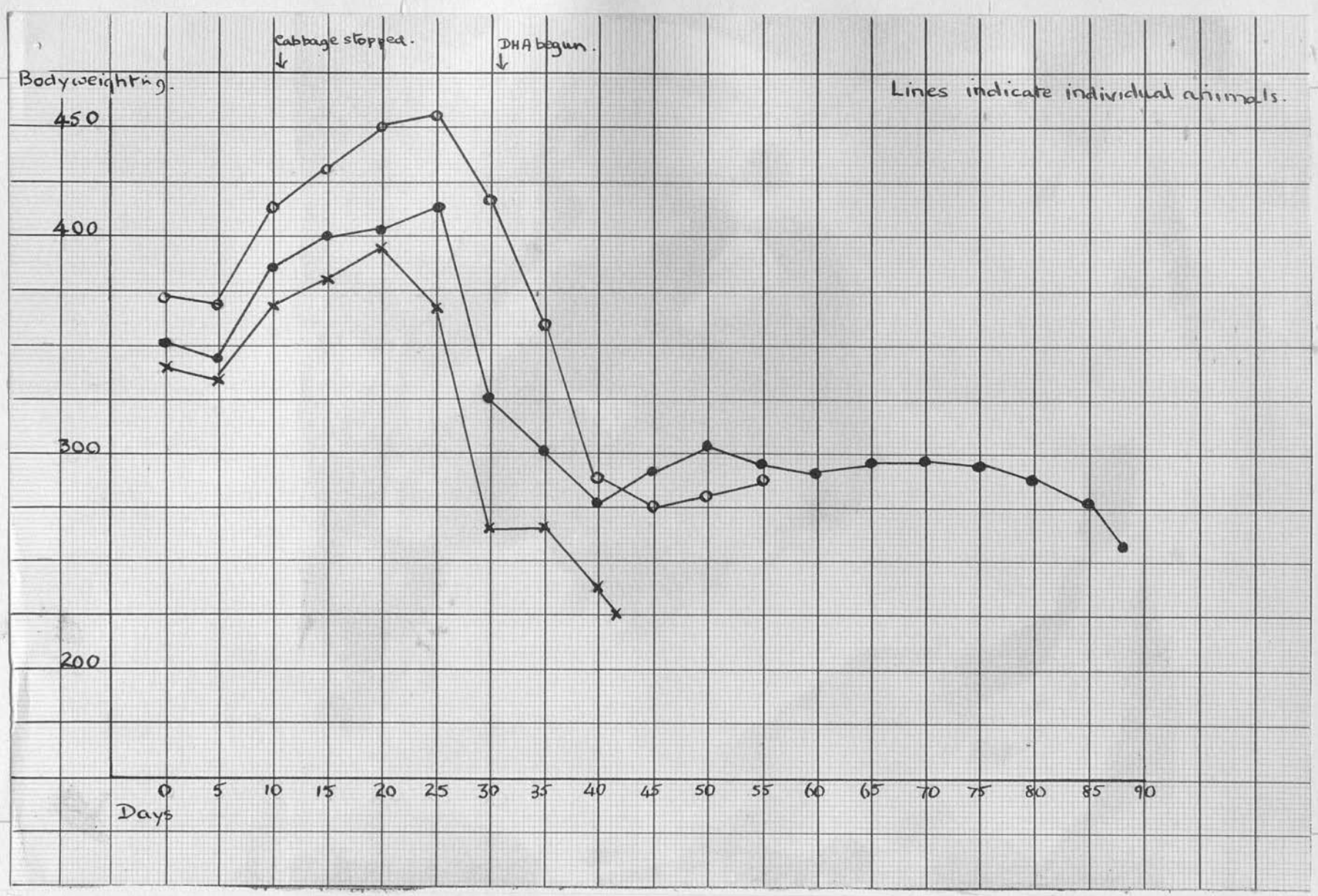


Figure 33 . Effect of 60 mg. DHA (Roche) by injection on the body weights of male guinea pigs maintained on a scorbutic diet.

and then subsequently a fall and death. Survival is much longer than normally occurs in the absence of an antiscorbutic substance.

3). In the terminal stages, all except pig F. showed marked flexion of the terminal phalanges - particularly pigs A, D and H. There was also partial paralysis of the hind legs, and these animals closely resembled those seen when DHA (Boots) was administered. There were no signs of jaundice.

4). At autopsy, every animal except C. showed very gross fatty degeneration of the liver. Sections showed the degeneration was greater towards the centre of the lobules.

The joints did not show evidence of scurvy; there was, however, a tendency for the epiphyseal lines to be a little narrower than normal, but the regular arrangement of the epiphyseal cells was maintained. Individual epiphyseal cells appeared larger than normal, and the matrix was very dense. There was no splintering of the bone. The marrow contained more fat cells than usual. Haemorrhage was seen in the gums of pig B, only, and did not occur elsewhere in any animal.

The urinary 17-ketosteroids in mg/24 hours/animal were determined in a pair of animals which had been receiving DHA (Boots) 60 mg/day per os for two months. Determinations were on two consecutive days.

	Urinary 17-ketosteroids.	
	60 mg. ascorbic acid per os.	60 mg. DHA per os.
Day 1	0.328	0.161
Day 2	0.192	0.266

There is no increased excretion of urinary 17-ketosteroids during treatment with DHA.

THE EFFECT OF DEHYDROASCORBIC ACID IN MICE.

Since the Physicians wished to inject dehydroascorbic acid (Roche and Boots) into patients, a toxicity test was performed on mice. For each compound a group of three mice were each given a third of a gramme by mouth on each of three days and then killed with ether on the fourth day. The animals were kept in cages with a glass bottom and the dry substance was mixed with a little powdered cube and placed in the bottom of the cage. By the afternoon the mixture had been eaten and the animals were then fed with a cube diet in the normal way. Water was supplied ad lib. After the first dose the mice began to adopt a hunched up position and developed some partial paralysis of their hind legs. The attitude was very similar to that seen in guinea pigs treated with dehydro-ascorbic acid. At autopsy the livers showed a moderate degree of fatty degeneration, particularly towards the centres of the lobules, in the mice treated with DHA (Boots) but were normal in those which had received DHA (Roche).

In view of the gross fatty degeneration produced in the guinea pigs and the somewhat milder fatty degeneration produced in the mice in a space of three days, the problem of the administration of the substance to patients was approached with great caution and the clinicians decided that it could only be administered for a period of a few days.

DISCUSSION.

Up to the present time ACTH still has to be assayed biologically, and it is most important that the bioassay should reflect the clinical response likely to be attained in the human patient. The present position is very unsatisfactory as there is no clear cut answer concerning the best method of assay, and indeed it is not yet known whether "ACTH" consists of one or several principles.

The ascorbic acid depleting assay of Sayers et al (1948) is the most widely employed, though its use is being questioned. For example, Kaine (1952) examined five ACTH preparations in male medical students. The human response was measured by the fall in eosinophils and rise in urinary 17-ketosteroids and the Sayers' test was used for bioassay. He found that there was an inverse relationship between the animal assay and the human response, so that the two preparations which gave the greatest human response, in animals showed the least.

Assays of ACTH may be divided into two groups. There are those in which the hormone acts directly without the intervention of adrenal steroids; these include the depletion of ascorbic acid and cholesterol, and adrenal weight maintenance and repair. The second group are dependant upon the formation and secretion of adrenal steroids and include the fall in eosinophils and lymphocytes, involution of the thymus and lymph nodes, and the healing test described in this thesis.

It has been shown that the ability to maintain adrenal weight does not parallel the power to deplete ascorbic acid

(Reinhardt& Li (1951), Young & Stacke-Dunne (1951), Dixon, Moore, Stacke-Dunne & Young (1951), Dixon, Stacke-Dunne & Young (1951) and Moyer, van der Scheer, Ritter, Tesar, Logan, Oleson & Cox (1952)). Similarly ability to deplete ascorbic acid does not parallel the eosinopenic activity (Reinhardt, Hungerford & Li (1951), Reinhardt, Geschwind & Li (1951), Hungerford, Reinhardt & Li (1952 a,b)). Hungerford et al (1952 b) drew attention to the fact that preparations can have ascorbic acid depletion activity and no eosinopenic activity, but the converse has not been found so far. Li (1952) has also shown that the ascorbic acid depleting power does not parallel the power to deplete adrenal cholesterol, to involute lymphoid tissues, to depress the thoracic duct lymphocytes or to form glycosuria.

As Reinhardt et al (1951 a) have pointed out, the overall evidence for the existence of more than one ACTH principle is indeterminate and discrepancies between various methods of bioassay may arise because the methods of administration in the tests are different, various preparations have different rates of absorption, and the time response relationships between various biological phenomena may not have been properly appreciated.

Talbot, Wood, Campbell, Christo & Zygmuntowicz (1951) postulated that there was more than one kind of ACTH, because in the human patient corticoid and ACTH production are stimulated to various extents. This however, leaves out the fact that the variable factor may in fact be in the adrenal gland.

Rheumatoid arthritis involves mesenchymal connective tissue in many parts of the body. It is characterised by 1) granulation tissue formation which leads to an overgrowth of fibrous tissue and hence destruction of functional tissue, 2) necrosis which commences as a fibrinoid degeneration, and passes into liquefaction and cavitation sometimes, and 3). round cell infiltration; lymphocytes predominate but variable numbers of plasma cells, histrocytes, and polymorphs also occur. The emphasis on each of the three components varies in different parts of the body (Gibson (1951)).

Histological studies of the changes in rheumatoid arthritis and periarteritis nodosa by ACTH and cortisone have been made. Hench, Slocumb, Polley & Kendall (1950) performed a biopsy of synovial tissue before and during the treatment of rheumatoid arthritis. Treatment always reduced the inflammation of the synovial membrane but never completely restored it to normal. Improvement was characterised by a reduction in the cellular reaction with decreased lymphocytes and plasma cells, a diminution in the deposition of fibrin, lessened oedema and evidence of fibrous healing. Kling (1950) also studied the effect of cortisone on the synovial effusion and observed similar changes. Fienberg & Colpoys (1951) had a patient with severe rheumatoid arthritis and scleral rheumatoid nodules. The patient was treated with cortisone for thirty one days and several nodules disappeared, the rest being reduced in size. Histologically during treatment the nodules showed involutional changes. There was a diminished

cellular exudate, an increased formation of multinucleated giant cells in the palisaded cell layer, increased cytoplasm in the palisaded cells with masked cytoplasmic vacuolization, central cyst formation and fibrosis. They concluded that cortisone accelerated the involutional changes that can occur spontaneously. Baggenstoss, Shick & Polley (1950, 1951) also examined the lesions of periarteritis nodosa in two patients before treatment and at autopsy after treatment with cortisone. In both, all signs of active inflammation of the arteries were absent at autopsy, and the healed lesions showed severe internal fibrosis which almost completely occluded the vessels.

In view of the reversal of the inflammatory changes which occur in rheumatoid arthritis it was thought that the mouse healing assay might be more closely related to the effect produced by ACTH and cortisone in the patient. It is therefore of interest that in eleven ACTH preparations assayed by both the healing test and the Sayers' test so far (Page 20), the potencies by the two tests have been virtually the same, (though our laboratory standard H.7911 has never been tested against La-1-A in the healing test). Work is in progress to compare the potencies obtained by these two methods with that obtained by an adrenal repair test, and also with the responses in the human patient. As Sir Henry Dale (1952) has pointed out the bioassay of ACTH must ultimately reflect its therapeutic effect.

It will be seen that the accuracy of the mouse healing test has fallen during 1952, and Mr. P. Armitage has commented on this; his remarks are quoted in the experimental part of

this thesis. It had already been noticed when attempts were being made to simplify the dosage schedule that inhibition was produced less often if the animals were cut later in the day, so that the gap in the night occurred earlier during the healing process. The wounds in assays performed during 1952 were made at midday, while in 1951 they were made at 10 a.m. This may possibly account for the reduction in accuracy. Certainly it would be easy to increase the number of mice if greater accuracy were required.

It is unfortunate that the test requires repeated injections, and that so far attempts to overcome this have been unsuccessful though some of the new bases being used by Armours for their long-acting ACTH may help. It has been shown by Sayers, Burns, Tyler, Jager, Schwartz, Smith, Samuels & Davenport (1949) and Greenspan, Li & Evans (1950) that ACTH disappears rapidly from the blood. Amongst others, Querido, Kassenaar, Coslings & Hymans (1951) have shown that repeated intramuscular or intravenous injections of ACTH are more effective in patients than single large injections. Similarly it was noticed in a patient of Dr. Prunty's (unpublished observation) that 40 mg. of ACTH daily divided into four doses of 10 mg. i.m. at six hourly intervals, gave about one tenth of the biochemical response elicited by the continuous intravenous infusion of 40 mg. of ACTH over a twenty-four hour period. It is obvious that in the healing test it is essential to maintain a continuous high concentration of ACTH.

The assay method lacks the specificity of that of Sayers et al (1948) but in view of its simplicity it is useful for some purposes - particularly the assay of preparations known to consist of ACTH. It may also be used as a screening test for compounds with a potentially cortisone-like action.

The experiments on the physiological factors affecting the response have proved most interesting. The change in sensitivity after hypophysectomy is in keeping with the well-known observation that following hypophysectomy the adrenal cortex becomes progressively less responsive to ACTH (Sayers et al 1948).

The physiological finding that the gonads were essential if ACTH was to cause inhibition was somewhat unexpected (Page 46). Three groups of adrenalectomized mice have shown inhibition of healing by ACTH;- a) those born at the beginning of the new breeding season, b) pregnant mice and c) those pre-treated with chorionic gonadotrophin.

It would appear that apart from one short period in the winter, the gonads and adrenals together are responsible for the effect produced by ACTH in the non-pregnant mouse. When the gonads have been under the influence of luteinizing hormone, their response is such that the adrenals are unnecessary for obtaining inhibition. It has been shown that although the presence of gonads and/or adrenals is necessary for the action of ACTH, cortisone will cause inhibition in the gonadectomised mouse. It therefore seems probable that one or more unknown compounds capable of causing inhibition are produced by the gonads in response to ACTH. These findings can

probably be correlated with those of McDermott, Fry, Brobeck & Long (1950) that the eosinopenic response to epinephrine in totally adrenalectomized male rats was not observed in male rats that were not simultaneously adrenalectomized and gonadectomized. They suggested that there was either an overlap in the effect of adrenal and gonadal steroids or stimulation by ACTH of adrenal cell rests in the testes.

Of the steroids examined for their effect on healing, only progesterone appeared to have any significant action. Taubenhaus & Amromin (1949) and Portugal, Lima, Rocha, Azulay & Silva (1951) found that testosterone and oestradiol would inhibit connective tissue formation in turpentine abscesses, but these hormones were administered over a very long period, so that various side effects - especially depression of the anterior pituitary gland - may have played a part.

There is a large amount of experimental evidence to suggest an adrenal-like action on the part of the gonads, and indeed from the adrenal cortex and gonad medulla have a common origin from the mesonephric blastema.

An excellent review on the adrenal-gonad relationship is available (Parkes (1945)) and only the more relevant facts will be referred to here. It has been well-established in different species including mice, that the functional state of the ovaries has a marked effect on the survival time after adrenalectomy. Thus during oestrus (e.g. Swingle, Parkins, Taylor, Hays & Morrell (1937), pseudopregnancy (e.g. Swingle,Parkins, Taylor & Morrell(1936)) pregnancy (e.g.Firor & Grollman(1933))

and after treatment with luteinizing hormone the survival rate is greater. Survival can also be prolonged by progesterone (e.g. Gaunt & Hays (1938), Greene, Wells & Ivy (1939)). Pfeiffer & Hooker (1940) have shown that these facts are true for mice, and in connection with our own healing experiments, it is interesting that these authors did not find an increased survival rate in mice pretreated with pregnant mares serum gonadotrophin and then adrenalectomised. A long and interesting series of experiments have been carried out by Hill and his colleagues on the life-maintaining properties of grafted ovaries (see Hill (1937, 1944, 1948), Hill & Gardner (1936), Hill & Strong (1938, 1940)). He found that if ovaries were grafted into the ears or testes of male mice, or autografted into the ears of female mice, they would maintain life in the complete absence of all adrenal cortical tissue. Removal of such grafted ovaries from adrenalectomized mice resulted in their death. Such ovaries usually contained clusters of "lutein-like" cells. These were not cell rests or normal lutein cells or cells of normal corpura lutea. They were in fact very similar to the cells of the adrenal cortex. Ponse (1948) has shown that such ovaries have an androgenic function. Hill was of the opinion that the lower temperature of the ear or scrotum was essential for the development of such an androgenic function, but more recently Katsh (1950) has shown that if ovarian grafts are placed in the seminal vesicles of castrated or castrated + adrenalectomized rats, they will largely prevent the regressive changes following castration.

Thus a lower temperature does not appear to be essential. Prevention of regressive changes was also apparent if grafts were placed in the testes, but not if placed in the thyroid, pancreas, foetal membranes or placenta. Similarly changes could be obtained with testosterone crystals, but not with cholesterol, oestrone or progesterone. Microscopically active ovaries often exhibited varying degrees of luteinization.

A seasonal variation in the survival after adrenalectomy has been reported. Bülbring (1937) found that the amount of cortin required to maintain adrenalectomized drakes varied with the time of year and was correlated with the size of the testes. Parkes (1945) suggested that after the breeding season the testes might elaborate substances that supplemented corticosterone. Similarly Britton (1930) found that the North American marmot could survive adrenalectomy when hibernating but died at the time of awakening. Cyclical variation in the gonadotrophin content of the pituitary gland has been shown by Friedman & Friedman (1939) and Novelli (1942) with rabbits and toads respectively. Such reports are in agreement with our finding of a cyclical change in the response of the ovary.

Changes in the adrenal cortex during the reproductive cycle can be correlated with ovarian physiology and adrenal cortical function. In this connection the mouse is of particular interest as the adrenal contains an x - zone. It is present in males and females until the age of five weeks, when it begins to degenerate in the male. It continues to grow in

the unmated female until puberty and then gradually degenerates and disappears before the end of the reproductive period. If the mouse becomes pregnant however it degenerates during the first half of pregnancy. A secondary x - zone arises post-puberally in castrated male mice. Much of the original work was done by Howard - Miller (1927) and Deanesly (1928). More recently it has been elaborated by Chester Jones (1949) who believed that the survival of the x - zone was dependant upon a gonadotrophin, possibly luteinizing in nature.

Literature on the changes occurring in the guinea pig adrenal have been reviewed by Parkes (1945) and show that the adrenal cortex particularly enlarges during pregnancy, a fact that may be important in connection with the high 17-keto-steroid excretion by pregnant guinea pigs in response to ACTH.

Other physiological findings too indicate that the gonads exert an effect on the adrenals. Gonadectomy influences the size and structure of the adrenals, and Woolley (1950) has described how certain strains of mice are liable to develop nodular cortical hyperplasia or adrenal cortical carcinomata after gonadectomy. It has also been shown that changes in the adrenal cortex can be induced by ovarian hormones e.g. Kimeldorf & Soderwall (1947).

It is interesting to speculate on the nature of the ovarian hormone produced in response to ACTH. Since C.G. alone is an insufficient stimulus to the ovaries to cause

inhibition of healing, it would appear unlikely that endogenous progesterone is responsible. Also the ovaries of the untreated adrenalectomized mice which showed inhibition of healing in response to ACTH appeared to contain no progesterone - secreting tissue. Direct experiments with progesterone showed much less striking effects than with ACTH. It is possible that the ovary could produce a steroid of the C.20 - C.21 ketol type. The fact that the gonads were essential for ACTH inhibition is difficult to fit in with this hypothesis unless it is assumed that insufficient hormone was excreted in response to ACTH by the adrenals alone and that the response was an additive one. It is possible that some degree of synergistic action of secreted hormones is involved. So far, attempts to demonstrate histologically any cells that might account for the effect, have failed. In this connection, Watteville, Borth, Mach & Musso (1951) gave ACTH to seven adults and ten children and could find no urinary pregnandiol as a result of therapy; though of course this does not rule out the possibility of very small amounts being excreted. There seems little doubt however, that varying changes in gonadotrophins in turn influence the gonads, and so determine, at any rate in part, the response to ACTH.

The direct <u>effect of ACTH and cortisone on the gonads</u> has been examined by some workers. Antopol (1950) found that following cortisone administration to mice, the testes showed retarded development with varying degrees of suppressed spermatogenesis and the seminal vesicles, prostates and

ovaries were smaller than controls. Similarly Baker, Schairer, Ingle & Li (1950) found that ACTH induced involution of the male reproductive system in rats. The testes became slightly reduced, 80% of them showed atrophy of the Leydig cells and the seminal vesicles showed changes similar to those following castration. They considered that the changes resulted from the negative nitrogen balance. Sohval & Soffer (1951) could find no effect of cortisone on the ovaries after two and a half days treatment in mice. There are of course, reports in the literature suggesting that ACTH has a stimulatory effect on the gonads (Davidson & Moon (1936), Davidson (1937), & Nelson (1941)), but it is conceivable that these workers were using impure ACTH. Selye (1946) has pointed out that stress in general will lead to regressive changes in the reproductive system of animals. The writer's own experiments on the short-term effect of ACTH on ovarian structure have given negative results.

Reports of an extra - adrenal action of ACTH from the laboratories of Selye have appeared during the course of this work. Jacot & Selye (1951) found that the injection of ACTH in rats without gonads or adrenals could result in stimulation of the growth of the preputial glands. They were not prepared to say whether it was due to ACTH or a contaminant but favoured its being ACTH, and subsequently (Jacot & Selye (1952)) confirmed the finding with a purified ACTH extract. They also showed that the effect was not due to gonadotrophin or prolactin. Selye (1951) demonstrated a thymolytic action in

adrenalectomized plus gonadectomized rats, and Selye and Jacot (1952) found that the thymolytic effect of cortisone in such animals was greatly enhanced by the simultaneous administration of ACTH.

During the course of this work and since, a number of papers have appeared on the inhibition of healing by cortisone; and a few on the prevention by ACTH of inflammation.

Ducommun & Mach (1950) produced a sterile peritonitis leading to multiple adhesions in rats by means of talcum powder. The adhesions were completely prevented by ACTH, though it had no effect once they had formed. Similarly, Coutu, Selye & Gareau (1951) showed that "topical irritation arthritis" in the rat could be prevented by pretreatment with ACTH. Green (1950) found that skin mitosis in the mouse was depressed for several hours by an injection of ACTH. The inflammatory lesions occurring in the hearts of dogs with Chagas' disease were shown by Gil, Perrin & Balcazar (1951) to be favourably influenced by ACTH but not completely erradicated. Similarly Woods & Wood (1951) observed that ACTH blocked the inflammation produced in rabbits' eyes in response to ACTH.

Cortisone suppresses healing in the mouse, rabbit, rat and man. Skin wounds were made in mice by Spain, Molomut & Haber (1950) and they found that 2.0 mg. cortisone/day by injection begun 24 hours before wounding, led to a complete suppression of all elements. After 24 hours there was almost no exudate or fibrin and few cells; as the days passed, only very sparse fibroblasts formed with little capillary formation and scanty ground substance though some did show epitheliazation. These workers found that once granulation tissue has

found cortisone had no fibrolytic action. In their experience cortisone did not effect the acute inflammation exudate forming in response to turpentine even though three days pretreatment were given. Baxter, Schiller, Whiteside & Straith (1951) used 3, 6 and 9 mg. cortisone by injection to inhibit increased wounds in rabbits. It depressed the growth of fibroblasts and capillaries and firm union of the epithelium was delayed; the rest of the animal's skin also showed atrophic changes. A similar effect was observed in the rabbit by Howes, Plotz, Blunt & Ragan (1950) using 6 - 10 mg. cortisone/kg. daily and by McManus, Cash, Carter, Alrich & Lehman (1951) using a dose of 40 mg/day. A somewhat similar result was observed in the healing of experimental fractures of the femurs of rabbits by Blunt Plotz, Lattes, Howes, Meyer & Ragan (1950). Healing of the fracture was greatly delayed especially after the fourth day with an irregular intercellular matrix, bizarre fibroblasts,and no bone trabeculation.

It has been shown that <u>cortisone will inhibit healing when applied locally</u>. Taubenhaus & Amromin (1949, 1950), Shapiro, Taylor & Taubenhaus (1951) and Taubenhaus, Taylor & Morton (1952) injected turpentine subcutaneously into rats and produced local inflammatory tissue. If cortisone was suspended in the turpentine the abscess was inhibited, though it did not inhibit the formation of an abscess in response to turpentine elsewhere in the body. As a result of cortisone,

granulation tissue was much thinner than round the control, the fibroblasts were smaller, flatter and less branched than normally, they did not run parallel to the capillaries, and more histiocytes and lymphocytes were present than normally, but there were fewer capillary buds. Meier, Schuler & Desaulles (1950) implanted pellets of compressed cotton subcutaneously in rats and the local application of cortisone inhibited granuloma formation. Lesions of the rabbit's eye can be inhibited by local cortisone (Woods & Wood (1951), and Jones & Meyer (1950)). Taubenhaus et al (1952) also showed that cortisone could act locally in hypophysectomised animals and Shapiro et al (1951) demonstrated that ischaemia or denervation did not effect the process. Hog adrenal extract applied directly to cutaneous wounds of rats will delay the closure of wounds (Baker and Whitaker (1950)) and the prolonged application of adrenocortical hormones to rat skin modified the histology of the treated area (Castor and Baker (1950)).

The action of cortisone can be antagonised by growth hormone (Taubenhaus et al (1952), Selye (1951)) and by DOCA (Taubenhaus et al (1952) and Aterman (1950)), but not by thyroxine (Taubenhaus et al (1952)).

The effect of other steroids on healing has been examined by other workers:-

a) Oestradiol: Taubenhaus et al (1952) showed that it would inhibit granulation tissue in response to turpentine by

a general systemic action. The experiment was confirmed by Portugal et al (1951) using oestradiol and testosterone together, cicatrization was improved in experimental vaginal wounds in ovariectomised rats by oestradiol, or progesterone (Sjovall (1948)).

b) Methyl androstenediol and testosterone: Taubenhaus et al (1952) showed that they inhibit granulation tissue if given systemically, but their effect is less marked than with oestradiol.

c) DOCA: provided the animal is pretreated for several weeks before turpentine is injected, then it promotes the formation of profuse abnormal granulation tissue (Taubenhaus & Amromin (1949), Taubenhaus et al (1952), and Pirani, Stepto & Sutherland (1951)).

There are obvious discrepancies between the results reported in this thesis and those in the literature, on the effect of steroids on healing. This may be because the writer's doses were not spaced correctly or not sufficiently high, but it seems more likely that the results of Taubenhaus and his colleagues and of Portugal et al (1951) were influenced by the fact that they administered the steroids for a considerable period before turpentine was injected e.g. Portugal et al (1951) gave 2.5 mg. testosterone propionate and 0.3 mg. oestradiol benzoate on alternate days for twenty days before injecting turpentine. Such a long pretreatment might presumably depress the anterior pituitary gland and so produce false results.

Various theories have been advanced concerning the mode of action of cortisone in causing inhibition. Taubenhaus et al (1952) have suggested that cortisone inhibits by counteracting the effect of growth hormone upon the target organ and that both growth hormone and cortisone act on factors responsible for fibroblastic proliferation. Similarly Baker & Whitaker (1950) thought the action was probably a direct one on the peripheral cells, especially fibroblasts, and Schneebeli (1950) found that four hours after cortisone was given to a mouse large acidophilic cytoplasmic inclusions were present in the fibroblasts. Castor & Baker (1950) suggested that the adrenal steroids might increase the resistance of the ground substance to depolymerization by hyaluronidase. Since the healing of fractured femurs in rabbits was not delayed until after four days Blunt et al (1950) thought there might have been poor nutrition of the tissues from an inadequate blood supply.

That cortisone will inhibit the skin-spreading phenomenon of mixtures of hyaluronidase in Indian ink in white mice was demonstrated by Anderson, Wiesel, Hillman & Stumpe (1951). This effect was reversed by the intraperitoneal injection of reduced glutathione but not by oxidised gluthathione. They therefore suggested that adrenal corticoids might possibly lead to a diminution in the availability of sulph hydryls and so to a blocking of various enzyme systems. ACTH certainly reduces the availability of sulph hydryl (Conn (1948)). Shacter & Entenman (1952) also suggested that cortisone and ACTH might

inhibit the availability of sylph hydryl groups required for the regeneration of injured tissues, and in 1952 found that cortisone and ACTH did not in fact effect plasma sulph hydryl levels in intact rats unless they were in a condition of stress from laparotomy or X - irradiation (Shacter, Supplee & Entenman (1952)). An interesting mechanism has been suggested by Green (1950). He observed that skin mitosis of mice was depressed for several hours following an injection of ACTH. Since the rate of skin mitosis is a reflection of mitotic activity in other tissues he thought this might be the method whereby ACTH causes a lymphopenia, general inhibition of body growth, eosinopenia and failure of granulation tissue formation. It might also depress mitosis in the cells of the reticulo-endothelial system from which antibodies are formed, and hence depress allergic inflammatory reactions.

The inability to inhibit healing in the guinea pig remains unexplained. Upton & Coon (1951) were unable to produce inhibition. They used a dosage of 4.0 mg. per 100 g. body weight and suggested that the lack of effect might be due to inadequate dosage, though it was enough to produce a fall in eosinophils and a reduction in adrenal size. Similarly Bangham (1951), and Magarey & Gough (1952) using doses of 2.5 and 10 mg/day/guinea pig were unable to produce inhibition, and Bourne (1952) observed no influence of cortisone on the tensile strength of wounds of scorbutic guinea pigs. On the other hand, Moon & Tershakover (1952) found that after thermal

injuries some effect could be produced by cortisone. They administered 5 mg. twice a day on the day preceeding and the day of the experiment. Less diapedesis, capillary haemorrhage and oedema occurred, and there was a definite though less effect on hyperaemia and leucocyte infiltration. They clearly demonstrated that the inflammatory reaction was definitely reduced. The experiments in this thesis agree with those of Upton & Coon (1951). The dosage of cortisone was as high as 50 mg. per day, and pretreatment was given for seven days before wounding, yet even so there was no inhibition, though oedema was probably reduced. However, Ungar, Damgaard & Weinstein (1951) were able to influence the swelling of the ankle joint which was produced by the injection of egg albumen into an already sensitised guinea pig, by ACTH and by cortisone. Cortisone was without effect in splenectomised animals. The swelling could also be decreased by sodium salicylate, heparin and certain anti-histamine compounds, and increased by DOCA, thyroid-stimulating hormone, large doses of thyroxin and splenin B.

There is still no conclusive evidence concerning the role of gonadotrophins in adrenal function, if indeed they play a part at all, and much of the evidence in support of a role is indirect.

It was claimed by Greep, Van Dyke & Chow (1942) that pure interstitial-cell-stimulating hormone only led to gonadal stimulation in hypophysectomised rats. Similarly Diczfalusy,

Holmgren & Westman (1950) found that massive doses of human chorionic gonadotrophin (C.G.) produced no adrenal changes in hypophysectomised rats. Cano & Botella (1950 a,b) had claimed that human C.G. did produce adrenal stimulation but their animals were intact. Chester Jones (1949) was of the opinion that the x - zone of the mouse adrenal was under a pituitary gonadotrophin probably luteinising in nature.

There are two possible modes of action for the gonadotrophins; either they can stimulate the gonads to produce sex hormones which in turn effect the adrenals, or it may be that sex hormones stimulate the pituitary to produce luteinising hormone (L.H.) which in turn directly stimulates adrenal function. The latter mode of action is favoured by Albright, Smith & Fraser (1942) and they explain the production of adrenal cortical hyperplasia and tumours in castrated animals by castration leading to increased L.H. secretion and hence to adrenal stimulation. Reifenstein, Forbes, Albright, Donaldson & Carroll (1945) also hold this view. They described an experiment in which 17-methyl testosterone (which is not excreted as a 17-ketosteroid) was administered to seven patients in which urinary 17-ketosteroids were believed to be of adrenal origin, and to two patients with Addison's disease. Since it resulted in a depression of 17-ketosteroids in both conditions, they suggested that the mode of inhibition was the same for adrenals and gonads, and was possibly attributable to L.H. Of the seven patients

however, six were women, and in view of the work of Huisn'tveld & Dingemanse (1952) this assumption is perhaps incorrect. Reifensten et al (1945) also quote other experimental findings. Thus Albright, Forbes, Fraser, Miller & Reifenstein (1941) observed that eunuchoid patients with high urinary F.S.H. had 17-ketosteroids within the limits for normal women, whereas those without high F.S.H. had low 17-ketosteroids. Reifenstein et al (1945) considered that this suggested that one hormone was stimulating both adrenals and testes. They also quoted an observation of Scott & Vermeuten (1942) that bilateral orchidectomy in patients with prostatic carcinoma usually failed to lower the 17-ketosteroids. If orchidectomy removed the inhibition to pituitary hormone production, this would lead to increased production and so to increased stimulation of the adrenals (though I do not see why such a hormone need necessarily be gonadotrophic in nature). A similar explanation could apply to the observation that young women show a rise in 17-ketosteroids for a year or so after castration or x - irradiation of their ovaries (Frank, Salmon & Friedman (1935)). Santos, Gomez & Botella (1951) observed the same effect in menopausal and castrated women.

More convincing experiments - giving opposite results - were performed by Nelson (1952) and Plate (1952). The former found that C.G. administration led to a rise in 17-ketosteroids in eight normal men. In a man with no testes and in a second in whom both testes were destroyed by fibrosis, C.G. led to no change in excretion at all. Thus this experiment produced no

evidence favouring the view that C.G. can act directly on the adrenal cortex. On the other hand, Plate (1952) injected two female castrates and a eunuchoid man with large doses of C.G. and found a rise in 17-ketosteroids in all three.

In view of the finding of an action of ACTH on the gonads, the possibility of a reciprocal action on the adrenals by gonadotrophins has interested the writer. So far it has only been shown that in castrated male and female guinea pigs, human C.G. or P.M.S. gonadotrophin have failed to influence 17-ketosteroid excretion (Page 138). In view of the high excretion observed in pregnant animals (Page 141) the problem is being pursued further to see whether combined administration of ACTH and gonadotrophin, or ACTH and a sex hormone will increase the response of the adrenal to ACTH. In this connection the observation of Pinto (1945) is of interest. Oestrogen alone did not increase adrenal hypertrophy in hypophysectomised rats, but if given along with ACTH the hypertrophy was greater than with ACTH alone. The problem is not of academic interest only since Paulsen (1950) found that pretreatment of children with pregnyl before ACTH, led to a response when formerly ACTH had had no effect at all.

In view of the close relationship between adrenals and gonads, the effect of ACTH and C.G. on testicular ascorbic acid was investigated, and an entirely negative result was obtained. Miller & Everett (1948) however produced a significant depression of the ascorbic acid of the corpus luteum in rats one hour after the injection of lactogenic hormone.

Cortisone and ACTH have not led to such deleterious effects on pregnancy in the writer's animals as the literature led one to expect. Glaubach, Antopol & Graff (1951) administered cortisone to pregnant mice and according to the length of time before parturition, the foetuses either died in utero with signs of retarded development and often autolysis, or died soon after birth. ACTH however begun 3 - 4 days produced no effect. Robson & Sharaf (1951) found that ACTH usually interrupted pregnancy in rabbits and mice, the effect was apparent in ovariectomised rabbits. Cortisone usually interrupted pregnancy in both species, and also in ovariectomised mice and rabbits and hypophysectomised rabbits. They concluded that these effects were not due to a contaminant or to any action on the ovary. It is therefore very striking that in the healing experiments on the guinea pig (page 41) pregnancy was not interrupted by very high cortisone dosage (50 mg./day for nine days) or by ACTH administration for seven days. When the animals were killed, the foetuses were still alive, and those near term showed respiration when the foetal membranes were removed. It may be that the guinea pig is immune to the abortive action of ACTH and cortisone reported on rabbits and mice.

It is not without interest that the foetal adrenals in the ACTH treated pregnant animals were smaller than the controls, suggesting that the maternal corticoids, but not injected ACTH,had crossed the placental barrier.

It is dangerous to draw too close an analogy between animal experiments and the findings in human patients, but the experimental results in this thesis may in fact explain some of the findings in Addison's disease. Patients with this disease may improve during the latter part of pregnancy, once the strain of morning sickness has terminated. Thorn, Dorrance & Day (1942) have commented on this and pointed out how very critical is the period immediately following delivery. Knowlton, Mudge & Jailer (1949) and Jailer & Knowlton (1950) have studied the steroid excretion of some of their patients during pregnancy. One was specially studied and she showed a rise in 17-ketosteroids and neutral reducing lipids during pregnancy, when she also showed eosinopenia in response to ACTH or epinephrine, though response was lost and the steroids returned to Addisonian levels in the early post partum period. They thought that adrenal cortical-like hormones were being elaborated by the placenta. They considered the foetal adrenals as a source but discarded the idea as the urine of newborn infants contains amounts entirely inadequate to account for the rise. The ovaries however do not appear to have been considered, though in the light of the experiments reported here it would be worth doing so. Prunty & Clayton (1951) have observed a patient with Addison's disease implanted with the maximum tolerated dose of DOCA who developed marked periorbital oedema at the commencement of the menses; in this patient the ovaries would appear to be the only source of increased "adrenal-like" steroids.

Clinically there appears to be some interaction between ACTH, cortisone and ovarian function. For example, Hench, Kendall, Slocumb & Polley (1950) observed that cortisone and ACTH sometimes induced amenorrhoea in their patients. Increased urinary F.S.H. was observed in the urine of nine out of twenty two patients as a result of treatment with cortisone and ACTH by Sohval & Soffer (1951). Attempts to correlate its appearance with other clinical findings were unsuccessful and its significance remains obscure. Urinary gonadotrophins were not altered in one patient studied by Mason, Power, Rynearson, Ciaramelli, Li & Evans (1948) and during two menstrual cycles of a patient studied by Sprague et al (1950). The reported increase in gonadotrophins is somewhat surprising, though the literature does contain reports of patients in which adrenal cortical tumours or hyperfunction have been associated with excessive urinary gonadotrophin excretion; e.g. McCullagh & Cuyler (1937) had a patient with Cushing's syndrome who had a positive Friedman test. Similarly increased gonadotrophins have been reported in three men with carcinoma of the adrenal cortex (McFadzean (1946), Reifenstein (1950) and Chambers (1949).

It should be remembered too that much of the work of Hench was stimulated by the observation in 1938 that twenty out of twenty two women with various forms of chronic arthritis, obtained striking relief during and for a variable period after pregnancy (Hench (1938)).

It has been shown conclusively (page 74) that the urinary 17-ketosteroid excretion in male and female guinea pigs rises during acute scurvy and reaches a peak in the terminal phases. In chronic scurvy (page 80) excretion remains low until the terminal phase.

Since this work has been carried out, two papers have been published independantly along similar lines. Banerjee & Deb (1952) also determined the 17-ketosteroid excretion in the urine of scorbutic and paired-fed normal female guinea pigs. Their results are in complete contradiction to those reported in this thesis. The normal level of excretion was 0.75 mg./ 24 hours (much higher than in the writer's animals) while in the scorbutic animals it was 0.77, 0.57, 0.56 and 0.51 mg. in the first, second, third and fourth weeks respectively, i.e. they excreted progressively lower amounts, and so these authors suggested that there was hypofunction of the adrenal cortex as scurvy developed. I am quite unable to account for the difference in our findings. Banerjee and Deb carried out their work in Calcutta, but the urine was collected over HCl so destruction as a result of an alkaline pH in a tropical climate should not have occurred, and after collection urine was stored in a refrigerator. Urine was however collected over seven day periods and it is not clear whether or not it was put in the refrigerator daily. They used acid hydrolysis and carbon tetrachloride extraction, followed by washing with water and 10% NaOH according to the method of Davison, Koets & Kuzell (1947). The Zimmermann colour reaction was employed

using a Lumetron photoelectric colorimeter with a 515 mμ filter, and a blank estimation was run too as suggested by Holtorff & Koch (1940). Tarantino (1950) obtained results apparently in agreement with those of Banerjee; he found that as the ascorbic acid in cardiac blood fell, so did the urinary 17-ketosteroids fall. The normal excretion in his animals was very high being 1.2 to 3.4 mg/24 hours, unlike the low levels in our normal animals. Presumably it is not inconceivable that the results of Banerjee & Deb (1952) and Tarantino (1950) are due to a strain difference.

Working quite independently, Nadel & Schneider (1951) published a short note describing an increase in the excretion of formaldehydogenic substances (F.S.) during scurvy, and later elaborated their findings (Nadel & Schneider (1952)). In growing male animals, the average excretion of F.S. in terms of desoxycorticosterone was 106 μg. In early scurvy this fell to 58 μg, rose to 140 μg. in middle scurvy, and to 387 μg. in late scurvy, with a peak excretion of 468 μg. These findings are therefore in very close agreement with those reported in this thesis for 17-ketosteroids. They also showed that high titres were immediately depressed by treatment with ascorbic acid.

The increase in 17-ketosteroids and F.S. does not appear to be a result of the stress from a diminished food intake. Nadel & Schneider (1952) found that starved animals on a daily supplement of ascorbic acid, showed no rise in F.S. until the loss in body weight was about 45%. And in humans too, Landau

Knowlton, Anderson, Brandt & Kenyon (1948) found that starvation actually led to a 50% fall in 17-ketosteroid excretion. This is important since Blumenthal & Loeb (1942) have pointed out that the hypertrophy of the adrenal glands seen in scurvy is very like that seen in underfeeding.

An experiment is described (Page 82) in which turpentine was injected subcutaneously into a pair of guinea-pigs. This stress resulted in a very small rise in 17-ketosteroids, vastly different from that seen in scurvy.

The experiments reported have shown that the source of increased 17-ketosteroids during scurvy is the adrenal glands (page 84). Adrenalectomized animals failed to show a terminal rise, but of course there is no absolute proof that death in these animals was due to acute scurvy, though the epiphyses showed early scorbutic changes and there were haemorrhages. Deprivation of ascorbic acid was certainly a major factor in the stress precipitating death. It may be that death resulted from deprivation of some other vital factor present in cabbage, since large supplies of cabbage are essential for successful survival of these animals. In the small number of experiments performed, castration of either sex did not lead to any significant change in 17-ketosteroid excretion during scurvy (Page 87). In adrenalectomized guinea pigs, the levels of 17-ketosteroids are so low, that it is doubtful whether the material is in fact steroidal at all.

As acute scurvy developed it has been shown (page 95) that the level of adrenal ascorbic acid has fallen, and the

and the findings were in agreement with those of Penney & Zilva (1946).

The role of ascorbic acid in the production of steroid hormones remains obscure, though a number of experimental findings suggest that adrenal activity continues though very small amounts are present.

Experiments reported in this thesis have shown that a rise in 17-ketosteroids occurs during scurvy and Nadel & Schneider (1952) observed a rise in F.S. It has also been shown that when the adrenal ascorbic acid is very low, the glands are still capable of responding to exogenous ACTH. (page 96). The response is additional to that provoked by the development of the scorbutic state, and within the limits obtained in healthy animals. There was an increase of 17-ketosteroids in the control animals comparable to that observed at a similar stage in untreated scurvy, and in excess of the basal level of 0.09 to 0.30 mg/day. These findings suggest that adrenal cortical secretion can undergo an increase in the presence of minimal amounts of ascorbic acid. It was also shown that the continued administration of ACTH throughout the development of scurvy could lead to an enhancement of adrenal hypertrophy (page 109), and a similar result has been noted by Eisenstein & Shank (1951).

There are other experimental findings to suggest that ascorbic acid is not obligatory for steroid production by the adrenals, particularly the observations of Zarrow & Zarrow (1950) and Zarrow & Baldini (1952) that in the duck and quail respectively response to ACTH is not accompanied by a change

in adrenal ascorbic acid though there is adrenal hypertrophy and hyperplasia. In the hamster too, the change in ascorbic acid is very small compared with that in rats and guinea pigs (Alpert (1950)). In the chick, Jailer & Boas (1950) showed that epinephrine or ACTH did not produce a change in adrenal ascorbic acid, though after four days administration there was hypertrophy of the glands; it was also shown that epinephrine caused a depletion of the sudanophilic material.

The observations of Long (1947) and Oesterling & Long (1951) also suggest that the relationship between adrenal steroids and ascorbic acid is not as close as some would believe. He showed that after fourteen to sixteen days on a scorbutic diet guinea pig adrenals contained only 4% of the normal amount of ascorbic acid, while the cholesterol is increased by 20%. Six hours after the injection of ACTH he found a fall in cholesterol and a lymphopenia, but no further loss of ascorbic acid in the gland which was in any case almost free of it. Vogt (1948) could find no consistent differences between the ascorbic acid content of the blood entering and blood leaving the adrenal cortex under conditions of increased activity.

There are in addition several clinical findings that may be interpreted in the same way. Thus Treager, Gabuzda, Zamcheck & Davisdon (1950) found that patients with clinical scurvy had normal eosinophil responses to ACTH and Daughady, Jaffe and Williams (1948) observed normal levels of corticoids in three scorbutic patients, with a fall following the administration of

ascorbic acid. Such clinical findings, though suggestive, are always somewhat unsatisfactory as there is no knowledge concerning adrenal ascorbic acid levels.

There are however, many workers who hold the view that ascorbic acid is in fact obligatory. Stepto, Pirani, Consolazio & Bell (1951) did not agree with Long (1947). They concluded from histochemical studies that there was an optimum ratio between ascorbic acid and cholesterol for normal steroid production, and that in the absence of adequate ascorbic acid, cholesterol could not be mobilised quickly.

In a long series of papers, Giroud (1940), Giroud & Ratsimamanga (1940), Giroud & Santa (1939), Giroud, Santa & Martinet (1940) have concluded that ascorbic acid favours the production of adrenal cortical hormones and improves their utilization. They consider that a sufficiency of vitamin C is necessary for the excretion of cortical hormones, that a fall in vitamin C is accompanied by a fall in the suprarenal functional capacity and that vitamin C and the cortical steroids are used simultaneously. The evidence that the concentrations of steroids and vitamin C in adrenal glands parallel each other was based, for the steroids, on biological assay. In this assay the partial contraction of chromatophores in certain fish was considered a specific response to cortical steroids. This is, of course, open to question, and no urinary steroid determinations were performed. They lay much stress on their considered opinion that the symptoms of scurvy in guinea pigs are similar to those of adrenal insufficiency, and that both

conditions show disturbances of water, salt, carbohydrate and protein metabolism. After the administration of cortical extract they claimed survival of scorbutic pigs for forty instead of twenty eight days, though, apart from less apathy and muscle atrophy, morphological differences were not evident. Lockwood & Hartman (1933) also noted the similarity between the symptoms of adrenocortical insufficiency and those of scurvy.

No prolongation of survival as a result of ACTH or cortisone administration in acute or chronic scurvy has been demonstrated. Indeed, there was a suggestion that ACTH may even have enhanced the haemorrhagic manifestations which tended to occur in unusual sites in these treated animals. The right adrenal gland in mg. to highest body weight in g. ratio was increased by ACTH and reduced by cortisone. In acute scurvy, cortisone did not influence haemorrhage, but it did reduce it in the chronic scorbutic animals. It would appear therefore that ACTH and cortisone do not influence the ultimate outcome of the disease at all, though minor changes may be seen as a result of their use.

Careful perusal of all the papers published on this subject while this work was in progress, and since, has failed to find any reasons for the discrepancies. The papers published are summarised here:-

A. A beneficial effect has been claimed by the following workers.

ACTH

Hyman, Ragan & Turner 1950.

Eisenstein & Shank 1951.

Hughes, Swenson, Underbjerg & Hughes 1952.

Cortisone

Schaffenburg, Masson & Corcoran 1950.

Hyman et al 1950.

Herrick 1951.

Herrick, Mead, Egerton & Hughes 1952.

Bourne 1952.

Cortical extract.

Lockwood & Hartman 1933.

Lockwood, Hartman & Hartman 1933.

Ratsimamanga 1944.

B. No effect at all has been claimed by:-

ACTH.

Upton & Coon 1951.

Cortisone.

Pirani, Stepto & Sutherland 1951.

Upton & Coon 1951.

Wolbach & Maddock 1952.

Cortical extract.

Vars & Pfiffner 1934.

Grollman & Firor 1934.

Svirbely & Kendall 1936.

C. ACTH aggravates the lesions:-

Nigeoi - Dureiul, Rabinowicz & Ratsimamanga 1951.

Hughes et al 1952.

Nigeoi - Dureuil et al (1951) found the injection of ACTH intensified the lesions of the alimentary tract and it

was thought that this was probably so in the writer's animals. Hughes et al (1952) thought that ACTH - treated animals developed arthritis a few days earlier. All workers except Wolbach & Maddock (1952) are in agreement that cortisone restores the adrenal body weight ratio to normal. It is not apparent why Wolbach & Maddock's finding was different. Otherwise their findings are entirely in agreement with ours, and they too were unable to demonstrate beneficial effects on any organ in acute scurvy. Some of the published papers are very scrappy and few details are given, but Hyman et al (1950) claimed that cortisone and ACTH reduced the haemorrhagic manifestations and prolonged survival by eight days. Herrick et al (1952) found that their treated animals remained almost normal with no painful joints and no changes in bone structure, and survived 17 - 45 days compared with 12 - 19 days in control animals. One wonders from their paper, why in fact their animals died at all; also the period of survival is very variable and one would welcome more details.

It was not possible to correlate the outcome of treatment with the doses of cortisone and ACTH used, or with the original body weights. It was thought that the varying scorbutic diets used might account for the differences, and this factor has been examined. Adequate dietary details are not available in every paper.

A. Diets in experiments reporting beneficial effects.

Lockwood & Hartmann (1933).

Whole milk powder dried at 110° C for 3 hours. 30%

Butterfat 10%

Rolled oats 39%

Bran 20%

Sodium chloride 1%

Schaffenburg et al (1950)

(a chronic scurvy diet).

This comprised orange juice and the basal diet of Rinehart & Mettier 1933. (This paper contains no details and attempts to track down the diet have failed).

Hyman et al (1950)

Soya beans 50% (autoclaved at 15 lbs, for 45 minutes).

Rolled oats 29%

Dried milk (klim) 10%

Brewer's yeast 4%

Butter 5%

Calcium carbonate 1%

Sodium chloride 1%

The ingredients were moistened with water after grinding, rolled into thin sheets and dried in an incubator. Filter paper was provided as roughage.

Eisenstein & Shank (1951)

Bran 24%

Crushed oats 26%

Weatings 21%

Salt mixture 5%

Fish meal 8%

Herrick et al (1951, 1952).

Rolled oats, soybean meal, Lederle fortified supplement, animal protein factor, vitamins A and D, alfalfa pellets in which vitamin C was destroyed by heating and drying.

B. Diets in experiments in which no effects were obtained.

Vars & Pfiffner (1934).

A proprietary dog food with added yeast, salt mixture and cod liver oil.

Grollman & Firor (1934).

Rolled oats 39%

Bran 20%

Skimmed milk powder 30% (heated in open trays at 110°C)

Fresh butter fat 10%

Sodium chloride 1%

Upton & Coon (1951)

Rolled oats 39%

Bran 20%

Skimmed milk powder 30% (heated in open trays at 110°C)

Fresh butter fat 10%

Sodium chloride 1%

Wolbach & Maddock (1952)

Soybean meal 36% (autoclaved at 15 lbs, for 1 hour).

Ground rolled oats 25%

Skimmed milk powder 20% (autoclaved at 15 lbs for 1 hr).

Brewer's yeast 4%

Alfalfa meal 8% (heated at 100°C in shallow pans for 8 hours)

Peanut oil 5%

Calcium carbonate 1%

Sodium chloride 1%

1 cc. of cod liver oil twice a week.

Some of these diets are very similar but none are exactly the same in experiments where different results are obtained by two groups of workers. Crampton (1947) has pointed out that the dietary requirements are not fully known, and it may be that some unknown dietary factor is playing a part too. It would also be interesting to know whether for example the dried milk used in the diet of Hyman et al (1950) was completely free of ascorbic acid; only a very minute amount is necessary to produce chronic scurvy, and they found that haemorrhage was reduced - only seen in the writer's experiments in chronic scurvy.

It is interesting that Hyman et al (1950) have added an addendum to their paper, in which they state that further experience has shown that it is possible to produce severe toxic effects in guinea pigs with large doses of ACTH and cortisone, so that their protective action may not occur. Certainly the experiments in this thesis do not suggest that ACTH and cortisone are antiscorbutic.

Ascorbic acid is capable of preventing many of the biological effects normally arising as the result of stress. Dugal & Therien (1949) showed that the typical enlargement of

the adrenals which occurs in response to the stress of cold in rats and guinea pigs can be completely prevented by large doses of ascorbic acid, and they thought that ascorbic acid apparently played a compensatory part rather similar to that of one of the adrenal hormones. A series of experiments along somewhat similar lines have been carried out by Bacchus and his colleagues. They showed that pretreatment with ascorbic acid prevented the eosinopenia characteristic of the alarm reaction in the rat (Bacchus & Toompas 1951, Bacchus 1951) and in the mouse (Bacchus & Altszuler (1952)). They also showed that ascorbic acid could potentiate the gluconeogenic action of injected cortisone (Bacchus, Heiffer & Altszuler (1952)), and would prolong the haematological action of cortisone (Bacchus et al (1952)). Their conclusions are summed up in the paper of Bacchus, Heiffer & Altszuler (1952) in which they show that ascorbic acid alone does not alter leucocyte or blood glucose levels. It can however block the haematological effect of epinephrine but not the response to ACTH or cortisone. They therefore suggest that ascorbic acid may prevent the release of ACTH by the anterior pituitary, and since it is possibly synergistic with cortisone in some respects, this could explain the non-activation of the pituitary-adrenal axis in ascorbic acid-treated animals exposed to stress. Bacchus, Altszuler & Heiffer (1952) in a rather confused argument considered that the rise in 17-ketosteroids observed by Clayton & Prunty (1951) might be due to an absence of

ascorbic acid rather than to adrenal hyperactivity.

These experimental findings may possibly explain the observation of Kayahan (1952) that the administration of high doses of ascorbic acid for four days by mouth to humans, led to a depressed 17-ketosteroid excretion and a raised cortical excretion. He suggested that ascorbic acid can act directly on the adrenal cortex. The writer too, has a strange observation that may in fact agree with this; on several occasiona it has been found that the intravenous injection of ascorbic acid sometimes depressed the adrenal ascorbic acid of hypophysectomized rats. It is not an invariable finding and cannot be explained at present (the completeness of hypophysectomy is in no doubt).

Unlike Gallagher et al (1951) no significant amounts of material giving the purplish colour of the Zimmermann reaction have been found in rat or guinea pig faeces. Faeces were examined before and after treatment with ACTH, and cortisone by injection and by mouth. The amount of 17-ketosteroids undetected in our experiments because faeces were not routinely examined would appear to be extremely small. The absorption curves have already been described. Ashmore, Elliott, Doisy & Doisy (1935) administered testosterone - 4 - C^{14} to a rat and found one third of the activity in the urine and two thirds in the faeces, but no indication of the chemical nature of the metabolites was given.

The experiments with DHA reported here (page 147) have shown that both preparations have an antiscorbutic effect, though treatment with both preparations ultimately resulted in

death. When the dose was small survival was greatly reduced as compared with the high dosage. The animals did not show evidence of scurvy, but almost all developed very severe fatty degeneration of the liver. With the higher doses their growth curves showed increases to begin with, and then their weights began to fluctuate and they ultimately died; the curves were quite different from those seen in ascorbic acid-treated animals. Fatty degeneration was produced too, in the livers of the mice receiving DHA (Boots). No glycosuria was found in the guinea pigs. During the last few days of life most of the animals developed a similar appearance; they apparently had partial paralysis of their hind legs and the phalanges of their hind and fore legs showed extreme flexion together with loss of hair over the phalanges.

In view of the severe fatty degeneration of the liver the long-term treatment of patients is to be deprecated until more is known about these drugs. Dr. Prunty (unpublished observation) has given intravenous DHA to a rheumatoid patient for several days without any amelioration of the condition resulting. It is quite probable that the fatty degeneration results from an unknown contaminant, as DHA is difficult to make and highly unstable.

Evidence has gradually been collected, suggesting that cabbage may contain an unknown factor important in the diet of the guinea pig. The data may be summarised as follows:-

1). Provided an adequate quantity of cabbage is supplied, doses of cortisone as high as 50 mg./day for nine days have no

adverse effect on body growth in pregnant and non-pregnant females and males. Doses of cortisone as low as 1 mg. per day ultimately lead to death of guinea pigs if they are maintained on 2 mg. ascorbic acid per day by injection, and death also occurs on a daily ascorbic acid intake of 50 mg, when the cortisone dosage is 10 mg. The cause of death is not known.

2). Adrenalectomized guinea pigs will only survive if given cabbage and they usually have an excessively high daily intake. As soon as cabbage is replaced by large quantities of lettuce and celery, or adequate doses of ascorbic acid by injection they lose weight and die within a few days while being maintained on DOCA.

3). In three out of four experiments, the injection of ACTH or saline to adrenalectomized guinea pigs has increased their cabbage intake significantly during the injection period and for a few days thereafter. Cabbage contains little sodium in comparison with scurvy diet and it seems unlikely that they eat it to increase their salt intake.

In connection with these findings it is interesting to note those instances where marked flexion of the phalanges, loss of fur, and partial hind leg paralysis has occurred:-

1). Animals maintained on scurvy diet supplemented with the equivalent of 2 mg. or 60 mg. of DHA (Roche and Boots).

2). Two intact animals maintained on scurvy diet plus ascorbic acid by injection (10 mg/100 g) and given daily ACTH until they ultimately died.

3). One adrenalectomized animal maintained until death (at ten days) on DOCA and cortisone, and scurvy diet plus

ascorbic acid by injection (10 mg/100 g. body weight).

((2) and (3) were observed during experiments on ascorbic acid metabolism which are not reported in this thesis).

It is possible that these observations may link up with those of Wagendonk & Wulzen (1950). They described a condition in guinea pigs characterised by the development of stiffness of the joints, arteriosclerosis with necrosis and calcification of skeletal muscle and myocardium, and the deposition of calcium salts in the smooth muscle of the gastro-intestinal tract, in kidneys and in liver. They also showed loss of hair and dragged their hind legs when walking. This picture has been confirmed by Oleson, Vandonk, Bernstein, Dorfman & Subbarow (1947) and by Petering, Stubberfield & Delor (1948). Its existence has been denied by Kon, Bird, Coates, Mullin & Shepheard (1946). In dealing with the existence of an unidentified dietary factor it is reasonable to expect contradictory reports from different laboratories. This anti-stiffness factor is possibly a precursor of a compound normally synthesised in the animal body. It is present in cream, molasses and green vegetables and has been tentatively identified as a steroid by Ross, Van Wagtendonk & Wulzen (1949). Its formula was $C_{28}H_{460}$, it gave a precipitate with digitonin, had one alcohol group and two double bonds.

The writer's animals were certainly not showing the fully developed condition, but it is possible that it was a milder form. It is possible that animals maintained on scurvy diet and ascorbic acid could manufacture their own

anti-stiffness factor though this was not possible after prolonged ACTH treatment. It is also possible that the adrenalectomized animal cannot make it and must be supplied with it in cabbage. Intact animals on DHA and scurvy diet show the condition, possibly because ascorbic acid itself is essential. If it is steroidal in nature it might explain the high cabbage intake in adrenalectomised animals, with increased amounts following the stress of injection.

Crampton (1947) has pointed out that the nutritional requirements of guinea pigs are not completely known and no diet is wholly successful in producing growth and successful pregnancy unless some greenstuff is fed too. He has devised a diet as follows:- ground oats 15 parts, ground wheat 13 parts, ground dried beet pulp 25 parts, linseed oilmeal 12.5 parts, skim milk powder 15 parts, fishmeal 5 parts, brewer's dried yeast 10 parts, bone char 4 parts, salt (iodized) 0.5 parts, together with vitamins A and D in the form of fish, vitamin E as γ-tocopherol and vitamin C as ascorbic acid. Provided this diet is supplemented with fresh or dried long - stored grass clippings (which contain no ascorbic acid at all) 80% of pregnancies are successful. Without the clippings however, only 60% are successful. There would appear to be some important unknown factor in the herbage.

The relation of cortisone "toxicity" to diet has been investigated by Meites (1951, 1952 a, b). He placed young rats on a casein - glucose diet with added vitamins and salts, and compared the growth of untreated controls with those receiving 1 mg. cortisone acetate daily subcutaneously on the

basal diet, and also when supplemented with B_{12} or aureomycin and especially by a combination of both. The supplements largely prevented the reduction in thymus weight by cortisone, but not the reduction in adrenal size or fall in eosinophils. He concluded that the administration of cortisone had increased their requirements for B_{12} and other factors. He performed a similar experiment with 10% whole liver as a supplement and obtained an even better response, though this might have been due to its carbohydrate, fat and protein content. Ershoff (1951) performed similar experiments but found no effect with B_{12} though he confirmed the results with 10% whole liver and later (1953) showed that the protective factor was in the water-insoluble fraction of liver. 10% whole liver was without benefit in the mouse. Meites (1952 b) has suggested that changes in endocrine balance may alter the nutritional needs of the body. Oestrogen and thyroid were found by him to inhibit growth, though this was partially overcome by B_{12} or antibiotics. Similarly, Dumm & Ralli (1948) and Ralli & Dumm (1952) found that pantothenic acid increased the survival of adrenalectomized rats, and Pentz, Graham, Ryan & Klein (1950) were able to partially counteract the loss in thymus weight that occurs in thyroid-fed rats by liver or B_{12}. Similarly, Lotspeich (1950) found that the pantothenic acid requirement was greater when rats were treated with growth hormone.

The writer has also found that cortisone has a "toxic" action on guinea pigs if they are maintained on a scorbutic diet supplemented with ascorbic acid; in the presence of

cabbage this is not observed. In the particular animals which received 2 mg. ascorbic acid daily (page 123), this dose led to changes in the epiphyses characteristic of chronic scurvy. Cortisone apparently stimulated new bone formation, so it may have had a sparing action on the ascorbic acid. Pfander (1952) using odontoblasts as a criterion, concluded that 5 mg. of cortisone had an initial sparing action on the ascorbic acid requirement, but by the end of 56 days the effect disappeared and there was evidence of retrogressive changes in the odontoblasts. The cause of death in cortisone "toxicity" remains unsolved at present.

SUMMARY

1). Adrenocorticotrophic hormone inhibits the formation of granulation tissue in mice, and this fact has been used as the basis of an assay in which a quantal response is measured. Potencies by this method and that of Sayers, Sayers and Woodbury (1948) are in close agreement.

2). Physiological factors affecting the response of experimental wound healing to ACTH have been investigated in mice.

a). No gonadotrophic activity was detected in the sample of ACTH used, and pitressin tannate and chorionic gonadotrophin did not produce this effect.

b). Hypophysectomised mice became progressively and rapidly less responsive to ACTH.

c). The gonads were essential for inhibition in very young and in mature mice.

d). Adrenalectomised mice at the beginning of the breeding season, adrenalectomised pregnant mice, and adrenalectomised mice pretreated with chorionic gonadotrophin showed inhibition of healing by ACTH. Adrenalectomised mice pretreated with follicle-stimulating hormone or pregnant mares' serum gonadotrophin were not inhibited.

e). Cortisone acetate caused inhibition in intact and gonadectomised mice. Neither testosterone, oestriol nor oestradiol had any effect, but progesterone gave definite impairment under the conditions of the experiment.

f). It is suggested that in mice the gonads are capable of responding to ACTH under the influence of luteinizing hormone.

3). Cortisone does not inhibit healing in guinea pigs.

4).

a)Guinea pigs placed on a scorbutic diet showed a gradual increase of varying extent in urinary 17-ketosteroid excretion. This reached a peak in the terminal phases. No increase occurred in the absence of the adrenals, but the increase was unaffected by castration.

b). Daily administration of ACTH in acute scurvy failed to influence the fall in body weight or time of ultimate death.

c). Daily administration of cortisone failed to influence the ultimate outcome of acute and chronic scurvy. Cortisone prevented adrenal hypertrophy in scurvy, and in chronic (but not acute) scurvy it reduced the haemorrhagic manifestations.

d). In animals maintained on a dose of ascorbic acid leading to chronic scurvy, cortisone stimulated new bone formation at the epiphyses.

5). In the late stages of acute scurvy when adrenal ascorbic acid is minimal, there is still a normal response to exogenous ACTH as judged by a rise in urinary 17-ketosteroid excretion, over and above that due to developing scurvy.

6). Normal pregnant guinea pigs show a greater response to ACTH than non-pregnant ones. Urinary 17-ketosteroid excretion was not increased by gonadotrophin administration to castrated animals.

7). It has been shown that dehydroascorbic acid (DHA) is antiscorbutic. DHA administration leads to the development of characteristic features, which include fatty degeneration of the liver, flexion of the fore and hind feet and partial hind-leg paresis.

8). The dietary intake of adrenalectomised animals has been measured. The possible presence of an important dietary factor, other than ascorbic acid, in cabbage has been discussed.

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